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LESIONS IN THE TISSUES OF THE BODY FOLLOWING SULFONAMIDE THERAPY*

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NOT since the introduction of arsphenamine and neoarsphenamine many years ago, has a therapeutic agent received such a welcome and cordial reception as when the sulfonamides were introduced to the profession. Originally introduced to combat serious and grave infections caused by the streptococci, especially *Streptococcus hemolyticus*, the value of these drugs was soon established in pneumonia, meningitis, various bacteriemias, genitourinary tract infections, bacillary dysentery and any number of other disease entities. There is hardly a single specialty in the medical profession which does not make use of one or more of the sulfonamide drugs. Unfortunately, the laity has become too well acquainted with these drugs, looking upon them as a cure for any and all ailments, and are therefore using them haphazardly and indiscriminately. Much harm has resulted in many instances from such usage.

As a matter of historical interest, one of the sulfonamides, sulfanilamide (para-amino-benzene-sulfonamide) has been known for many years. Gelmo,[1] working on the chemistry of azo dyes, made it synthetically in 1908. Eisenberg,[2] in 1913 called attention to the bactericidal properties of certain azo dyes. Among the various azo dyes tested were pyridium, chrysoidine, and scarlet red. In 1935 chemists working under Domagk[3, 4] produced prontosil I and prontosil II. Prontosil itself possesses poor bactericidal properties, but when its molecule is broken down in the body, a potent product, sulfanilamide, is set free. Trefouel,[5] and Fourneau[6] in France confirmed the contention that the azo dye upon being broken down in the body liberates sulfanilamide. Interest in these

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new drugs first developed in America in 1936. The work in this country by Mellon,[7] and Long and Bliss[8] is well known.

Nothing is definitely known about the therapeutic action of the sulfonamides. Theoretically, it may be assumed that the drug acts by: 1. A neutralization of bacterial toxins. 2. It stimulates the cellular and humoral defensive mechanism of the host against the infectious bacteria. 3. It acts as a germicide and destroys the invading and circulating bacteria. This is unlikely since it has been shown by Selbie[9] that simultaneous administration of para-amino-benzoic acid and sulfanilamide in experimental animals does not prevent a fatal outcome, the action of the latter being antagonized by the former. One molecule of para-amino-benzoic acid inhibits the action of 23,000 molecules of sulfanilamide. Janeway[10] has shown that the addition of small amounts of para-amino-benzoic acid to blood cultures inhibits the action of sulfanilamide and thereby permits the bacteria present to grow and flourish. This would seem then that sulfanilamide acts merely as a bacteriostatic agent, giving other defensive forces of the body an opportunity of assisting them in ridding the body of infection.

Many articles have appeared in the medical literature extolling the virtues of the various members of the sulfonamide group of drugs in the treatment of different infectious diseases. Practically every medical program offers one or more scientific papers describing new uses or discussing clinical results obtained with these drugs. As so often happens with new therapeutic agents which appear to accomplish unusually favorable results, no thought is given at first to the fact that in some instances they may also do considerable harm. This has also been true of the arsphenamines, cinchophen, atabrin, and many other drugs when they were first introduced. It is true that all new drugs are at first thoroughly controlled for toxicity and other properties. These tests are carried out, in the majority of instances, on normal experimental animals. Let us not lose sight of the fact that an experimental animal often reacts differently to a drug than does a human being. Of more importance is the fact that in the instance of the patient extensive injury may already have occurred in the important parenchymatous organs as a result of serious infectious disease. Naturally a drug which itself may be toxic adds insult to injury in such an instance.

Recently numerous reports have appeared in the medical literature describing unfavorable reactions and even death as a result of sulfonamide therapy. Various types of skin lesions, nephritis, focal necrosis, and acute yellow atrophy of the liver, involvement of the central nervous system, myocarditis, anemia, leucopenia, and various other conditions have been described.

Lesions of the skin appear as one of the most frequent complications and may be a manifestation of an allergic or toxic manifestation or otherwise as a drug idiosynerasy. Wien and Lieberthal[11] describe a pemphigus, foliaceous-like eruption following the use of sulfanilamide and sulfapyridine. Weinstein and Domm[12] report a case of a patient with pneumonia receiving a total of 15 Gm. of sulfathiazole. The patient developed a severe, acute exfoliative dermatitis. "Other than an overwhelming toxemia resulting from the widespread and severe involvement of the skin, no satisfactory cause of death was found." Volini, Levitt, and O'Neil[13] present a case report and discuss cutaneous and conjunctival manifestations of sulfathiazole intoxication. The severity of skin

lesions with extensive necrosis is well shown and discussed in a case report by Klingensmith.[14]

Lesions of the liver in sulfonamide poisoning have not received the attention they deserve. This is probably due to the fact that in the majority of instances such a diagnosis is arrived at only at autopsy. It has been our experience that

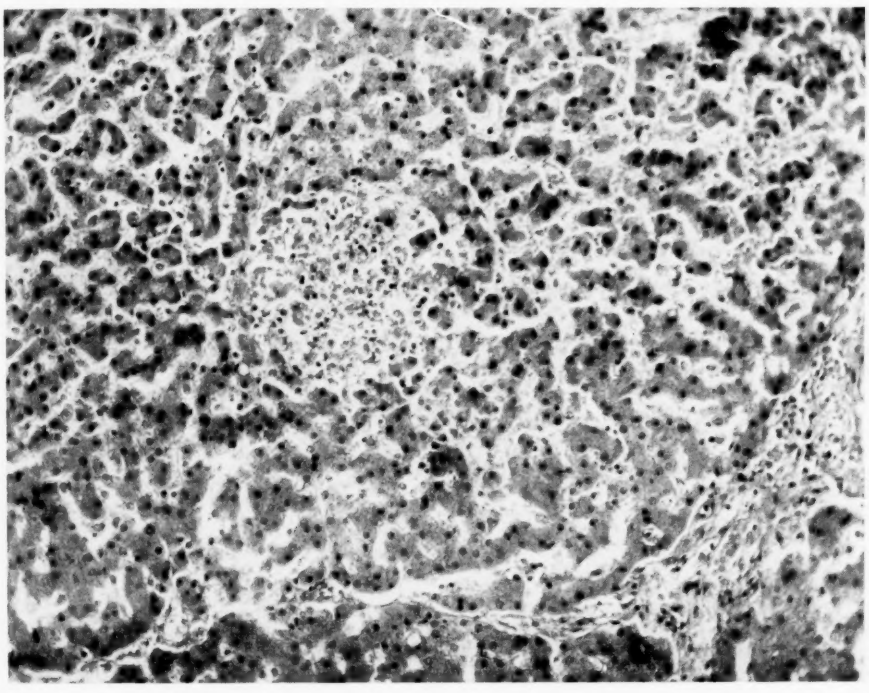

Fig. 1.—Photomicrograph of section of human liver. Note central area of focal necrosis. In other areas isolated liver cells likewise show necrosis. (×400.)

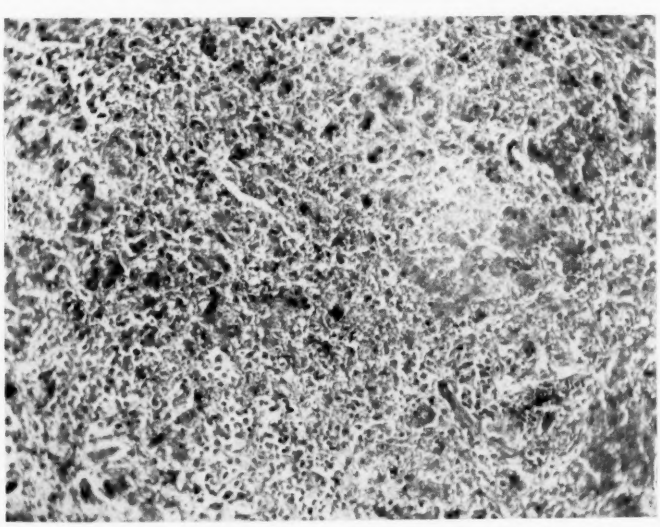

Fig. 2.—Photomicrograph of section of human liver showing extensive acute atrophy. Only a few isolated liver cells remain, and from the histology present it is difficult to tell that this is liver tissue. (×400.)

lesions in the liver are quite common and are frequently found at autopsy. The changes which we have noted at autopsy are severe cloudy swelling of liver cells, fatty degeneration, focal necrosis (Fig. 1), and in one instance there was encountered a case of acute yellow atrophy (Fig. 2).

Fig. 3.—Section of kidney showing tubules filled with blood cells and albumin casts. (×500.)

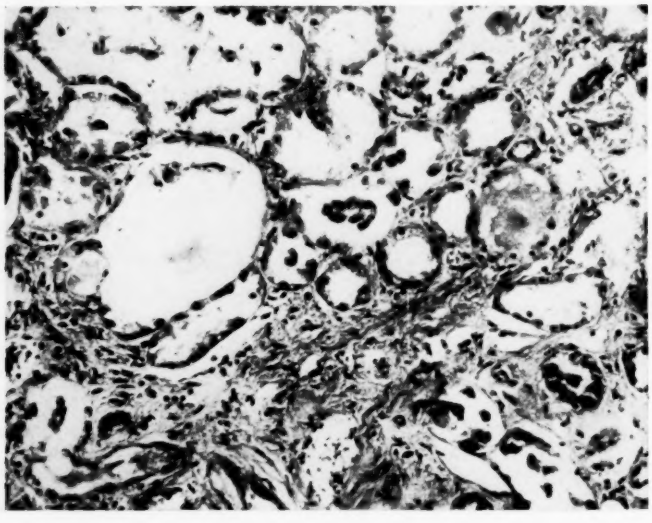

Fig. 4.—Section of kidney showing marked distention and dilatation of some of the tubules due to blockage by sulfapyridine crystals. (×500.)

The literature contains numerous reports of liver damage following sulfonamide therapy. Garvin[15] reports five cases of toxic hepatitis of patients who had primarily received sulfanilamide. A case of acute toxic necrosis of the liver following the use of sulfanilamide is described by Russell.[16] Watson,[17] in an excellent study, discusses the alteration of hemoglobin metabolism and hepatic function in a large series of cases receiving sulfanilamide and sulfapyridine.

Effects of these drugs upon the central nervous system are infrequently reported. One of the most recent reports is that of Little,[18] who describes the nervous and mental effects of the sulfonamides. Bieter and co-workers[19] carried out a series of experiments on chickens. Extensive peripheral nerve injury was noted. These investigators also present a case report wherein a patient developed deafness and loss of function of the right lower extremity following the use of sulfathiazole. These symptoms disappeared and function was restored after the drug was discontinued.

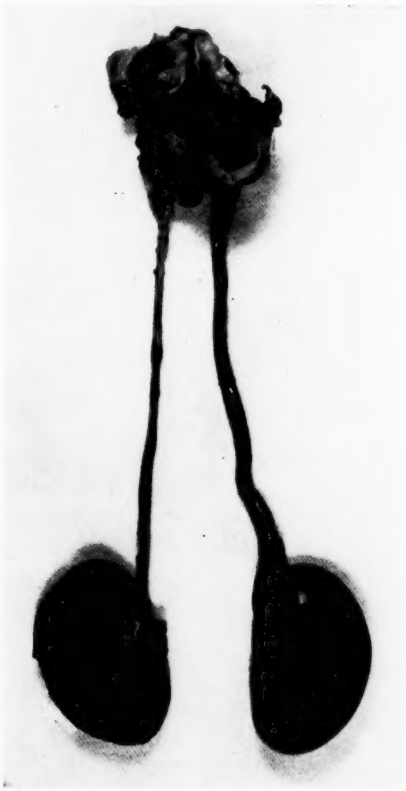

Fig. 5.—Gross picture of monkey's kidneys, ureters, and urinary bladder. Note marked ureteral dilatation and hydronephrosis on right due to blockage by sulfapyridine deposits. Ureter on left had been opened longitudinally and shows normal diameter of this structure.

Kidney injury is one of the most common complications encountered in patients receiving sulfonamide therapy. Instances of anuria, hematuria, nitrogen retention, and renal colic have been reported. Tubular casts we have noted are not an uncommon finding (Fig. 3). The insoluble acetyl derivative of these drugs forms crystalline deposits which may block the tubules (Fig. 4) or be so extensive as to cause ureteral blockage with hydronephrosis. It has been shown by Schwartz and co-workers[20] that crystalline precipitation is more likely to occur in acid urines. In a series of patients 28 per cent of those with alkaline urines showed crystalluria, while 70 per cent of those with an acid urine showed a similar precipitation. Alkalinization, therefore, seems an essential procedure to prevent precipitation and urinary tract blockage. The appearance of the

urinary acetyl derivatives of the various sulfonamides is well shown in the discussion by Lehr and Antopol.[21] It is usually not difficult to demonstrate crystalline depositions in tubular structures in frozen section preparations of kidney tissue. Passage of kidney sections through the various steps of the paraffin technique followed by hematoxylin-eosin staining dissolves out the sulfonamide crystals. Museum specimens of kidneys, ureters, and urinary bladders soon become worthless due to the fact that crystalline deposits of these drugs slowly dissolve in the Kaiserling solutions.

It was our privilege recently to examine the kidneys, ureters, and urinary bladder of a monkey which had died from an infectious disease. In treating the infection the animal had received large doses of sulfapyridine. At autopsy a large amount of crystalline material was found in the bladder and both ureters were completely blocked (Fig. 5) by crystal accumulations resulting in extensive bilateral hydronephrosis, undoubtedly hastening the animal's death. Excellent discussions of urinary tract changes are found in the articles by Winsor and Burch[22] and Antopol, Lehr, Churg, and Sprinz.[23]

Gross, Cooper, and Morningstar[24] have produced the experimental lesions of glomerulonephritis in rats with sulfapyridine. The lesions reported consisted of swelling and proliferation of the capsular epithelium, basement membrane thickening, hyaline degeneration of the tufts, endothelial proliferation of the tuft vessels, and adhesions of the tufts to Bowman's capsule.

Grave disturbances in the hematopoietic system may be encountered in sulfonamide therapy. This may take the form of a rapidly fatal acute hemolytic anemia, a fatal agranulocytosis, or less serious manifestations of these diseases. In the instances of agranulocytic angina, it is likely that these drugs may act in a manner similar to certain barbiturates, such as amytal, barbital, dial, or neonal. These are all "coal tar" derivative or "benzene ring" drugs, similar to the sulfonamides, and have a markedly depressing effect in some patients upon the leucoblastic centers in the bone marrow. It is needless to say that frequent leucocyte and erythrocyte counts and hemoglobin determinations during the course of therapy will forestall in many cases these serious complications. Spring and Bernstein,[25] Jennings and Southwell-Sander,[26] and many others report important findings from the hematologic standpoint in these cases.

An important lesion which is too infrequently mentioned is interstitial myocarditis. A review of the literature reveals the fact that attention is focused solely upon lesions of the kidneys, skin, liver, and hematopoietic system. It is very likely that changes in the myocardium have been overlooked in human autopsy material and also in experimental animals. It is hoped that the few articles appearing on interstitial myocarditis in patients receiving the sulfonamide drugs will stimulate further interest in this much neglected topic.

Our interest in interstitial myocarditis was occasioned by a perusal of the experimental work and autopsy study of human hearts by French and Weller.[27] We have noted histopathologic findings in human hearts similar to those reported by these authors. These consisted of eosinophilic cellular infiltrations between the muscle fibrils (Fig. 6), and in some cases forming collars about the small branches of the coronary vessels (Fig. 7). The cells were mainly large

Fig. 6.—Photomicrograph of section of human heart showing interstitial accumulations of blood cells. Most of these cells are large mononuclears with pink staining cytoplasm. There are present also some neutrophiles and a few erythrocytes. (×550.)

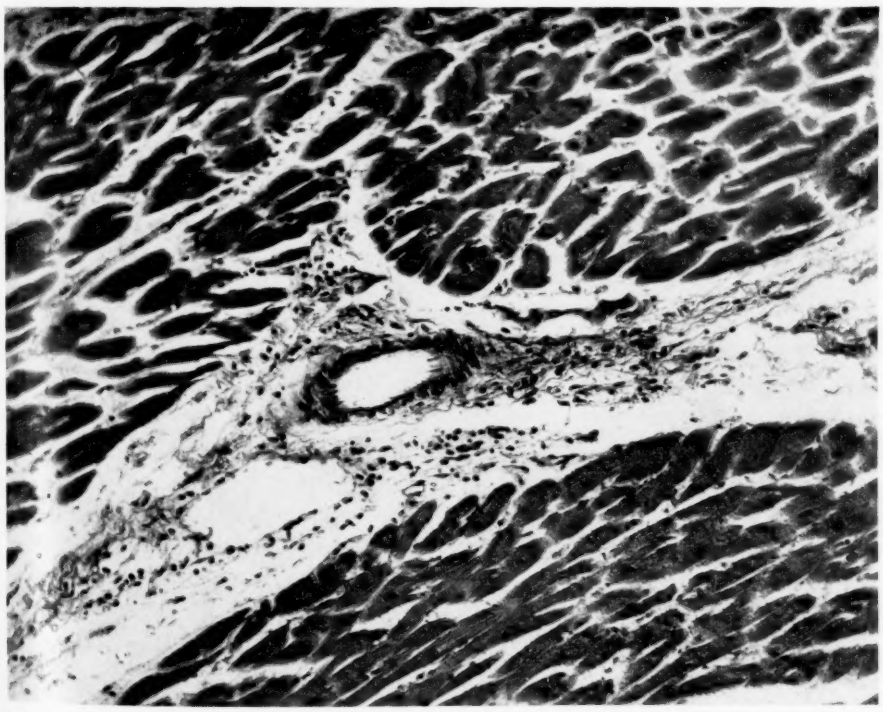

Fig. 7.—Photomicrograph of heart section showing perivascular infiltration with cells of a type similar to those described in Fig. 6 (×400.)

mononuclear cells with pink-staining cytoplasm and a considerable number of polymorphonuclear leucocytes, many with an eosinophilic cytoplasm.

In the study by French and Weller 126 cases, or 44.5 per cent, of the 283 receiving sulfonamide drugs showed a significant interstitial myocarditis. While the series studied by us so far is small, it probably will not reveal as high a percentage of interstitial myocarditis as reported by French and Weller.[27] In not any of our cases could the characteristic eosinophilic infiltrations be attributed to any other agent or cause than the sulfonamides.

Electrocardiographic changes were noted in a series of patients receiving these drugs, as reported by Scheinberg, and Ingle,[28] and Dozzi.[29]

In passing it might be appropriate to mention a word about the large number of deaths which followed the use of elixir of sulfanilamide in September and October of 1937. This whole matter is excellently reviewed in a Special Article[30] in the *Journal of the American Medical Association*. It should be re-emphasized that sulfanilamide was not to blame for these deaths, but its vehicle, diethylene glycol, a highly poisonous substance was responsible.

The tremendous popularity of the sulfonamides is shown by referring to the statistics of Drug Trade News, as quoted by Pruitt.[31] The manufacturers' sales in the United States in 1940 of sulfanilamide alone amounted to 543,802 pounds. What percentage of this was disposed of on physicians' prescriptions is difficult to say. The danger of the indiscriminate use of these drugs was pointed out in an Editorial[32] by the *Journal of the American Medical Association* as far back as 1937: "The startling news reports that the administration of sulfanilamide will 'cure' gonorrhea in forty-eight hours has led to some unpleasant results. Responsibility lies considerably with pharmacists who are willing to sell dangerous drugs to anybody over the counter. In one large city, hospitals have admitted young men with severe sulfhemoglobinemia resulting from self-medication with sulfanilamide. The physician must bear in mind the potential hazards of this drug."

CONCLUSIONS

A study of a large series of cases and a careful review of the literature reveals the important finding that any organ or tissue in the body may be affected through the toxic action of the sulfonamides. In what percentage of cases this occurs is impossible to say. Considering the popularity of these drugs and the tremendous quantity used, amounting to over one-half million pounds of sulfanilamide for 1940 alone, it is not surprising to note some bad effects and even deaths from time to time.

The immediate effects of these drugs upon the skin, liver, kidneys, and nervous system is well understood. It is not possible at this time to say what residual effects may be noted in the myocardium, liver, or other organs years later. Might such changes lead to chronic myocarditis or cirrhosis of the liver? Might they lead to bizarre blood dyscrasia? No one as yet knows the answer.

Greater precaution in the use of these drugs is advisable. Dispensing on physicians' prescriptions only would lead to proper control and discourage abuse and indiscriminate use by the laity. It is certainly not the intent to discourage a drug which has proved its merit. At times, as in grave pneumonias, meningitis, puerperal infections, and otherwise serious bacteriemias it may truly be a "life-

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saver." In cases of less gravity or in disease entities in which other therapy may be employed its usefulness is doubtful.

When sulfonamide therapy is indicated and necessary, certain precautions should be observed which may prevent disastrous effects from the toxicity of the drug. The amount of drug and duration of administration should be kept at a minimum consistent with beneficial results. Frequent checking to determine proper concentration of the drug in the blood is essential. Frequent erythrocyte and leucocyte counts and hemoglobin determinations with an immediate discontinuance of medication when there is evidence of important changes in these elements. An evaluation of renal function is necessary because a damaged kidney handles these drugs poorly. Progressive oliguria and hematuria may make continued administration of the drug hazardous. A continued high temperature after other signs of infection have disappeared may be entirely due to the drug. In a number of such cases observed the temperature has dropped to normal following the discontinuance of the sulfonamides. Alkalinization may prevent renal calculus formation. Electrocardiographic tracings may shed light on early myocardial changes following the use of these drugs.

An agent which is toxic for bacteria may in many instances be equally as toxic for the cells of the host and thereby increase the burden rather than lessen it. The normal tissues of the control animal, employed by the pharmaceutical firms, are affected to a lesser extent than the tissues and organs of a sick patient already the seat of cloudy swelling, fatty degeneration, etc., the result of bacterial toxemia.

Undoubtedly, with a check on the injudicious use of the sulfonamides, their discontinuance in diseases wherein the mortality is nil and where other forms of therapy are effective and close observation of the patient in whom their use is thought to be of decided benefit will result in fewer serious reactions and possible death.

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INFESTATION WITH STRONGYLOIDES STERCORALIS*

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HUMAN infestation with *Strongyloides stercoralis* in the Middle Atlantic states has been distinctly uncommon.[1] Infested persons in this section of the United States were usually carriers in whom original infestation was traceable to a sojourn in subtropical or tropical regions. Since infestation usually occurred by cutaneous or oral contact with stool from a carrier, it is reasonable to believe that cases occurred in persons who journeyed out of northern climates.

We were fortunate in having secured stool containing *Strongyloides stercoralis* larvae from a patient who had always lived in Maryland, except for a short visit to Virginia about twenty years ago. This case was of interest because the patient's infestation undoubtedly occurred in a nontropical region,

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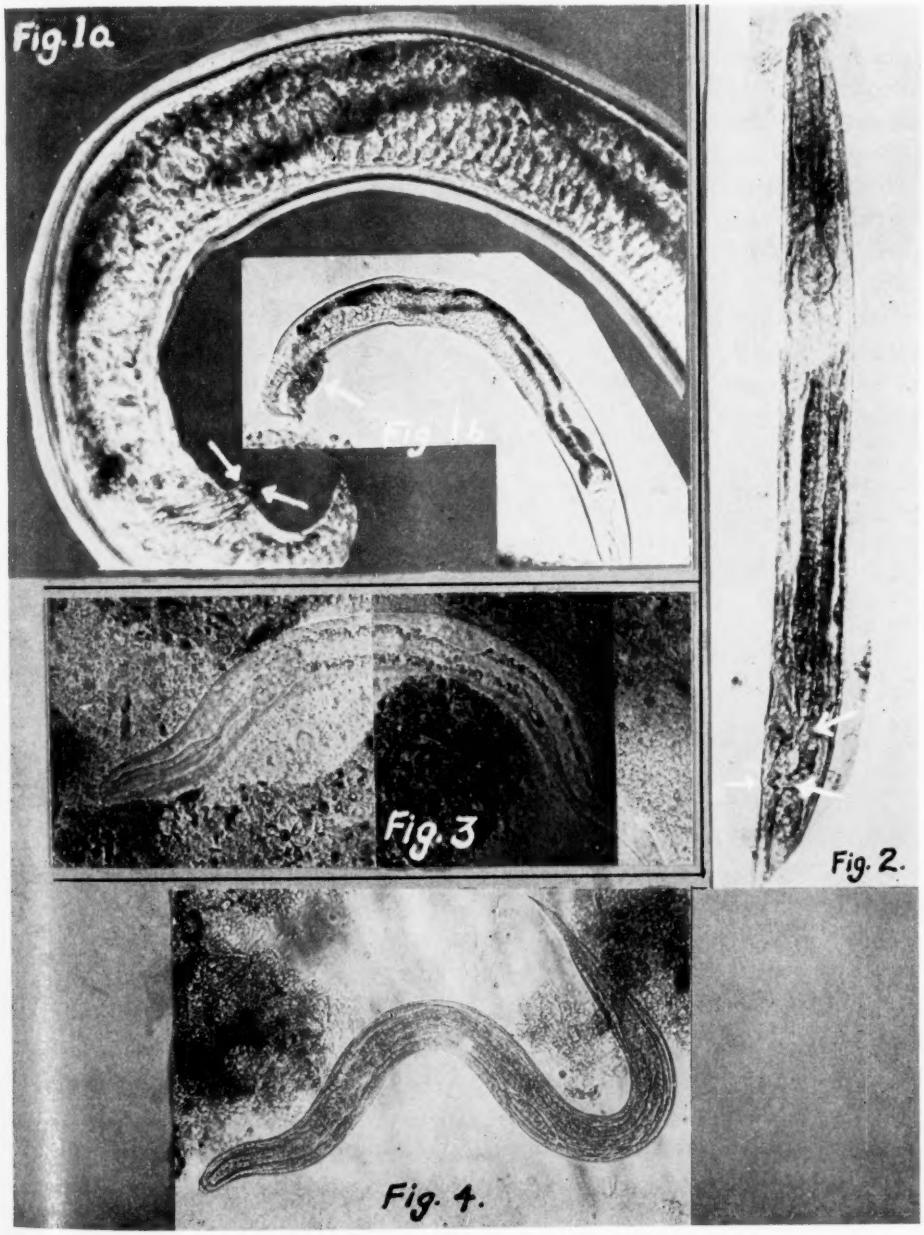
presumably from infested material from a carrier; and because of the opportunity presented of photographing the rarely observed and only recently discovered male form of the parasite.[2]

ABSTRACT OF REPORTED CASE

The patient presented no definite symptoms of *Strongyloides stercoralis* infestation and only a few very vague gastrointestinal complaints, such as flatu-



Figs. 1-4.—*Strongyloides stercoralis*, adult male and male and female larval forms.

lence, pyrosis, and constipation. He was a 51-year-old policeman who showed during the past year some anginal symptoms that were relieved by belching and morphine; his blood picture revealed a 6 per cent eosinophilia and a mild secondary anemia; and his electrocardiographic findings indicated some myocardial damage. Examination of the stool showed numerous *Strongyloides stercoralis* larvae which was the chief point of interest in this case.

DISCUSSION

In Fig. 1*a*, ×430 magnification, the male sexual organ of the adult form of *Strongyloides stercoralis was plainly seen.* The copulatory spicules and gubernaculum were indicated. In Fig. 1*b*, ×100 magnification, the fertilizing element of the male adult form was indicated at the exit of the sexual organ. Fig. 2, ×430 magnification, showed the male larva with the immature sexual organ indicated. In Fig. 3 and Fig. 4, ×430 magnification, the female larval forms of the parasite were indicated.

SUMMARY

A case is reported in which (1) infestation of *Strongyloides stercoralis* parasites was contracted in a relatively northern region; and (2) the rarely observed male larval and adult form of the *Strongyloides stercoralis* was found in the stool.

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THE EFFECT OF CINCHOPHEN ON BILE FORMATION*

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ON THE basis of the results of duodenal drainage experiments it has been reported that cinchophen is a choleretic in man.[1] In the anesthetized dog the drug has a very definite choleretic action[2]; it has no choleretic action in the rabbit.[3, 4] The effect of a single dose or repeated doses in the chronic biliary fistula preparation has not been studied. It was considered important to perform such an experiment because the drug, at least in large doses, is considered to be an hepatotoxic agent.[5]

METHODS

Five healthy biliary fistula dogs which had been carefully observed under dietary control for from two to three months were used. The bile was collected

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by the Rous-McMaster technique, except as otherwise stated. Three types of experiments were performed: (A) The animals were placed on a control period for from three to five days, the standard diet being fed twice daily without the return of bile or the administration of bile salts or drug. The volume output, and the cholic acid, cholesterol, and total pigment outputs were determined daily. Then the animals were given 1 Gm. of cinchophen in 0.5 Gm. doses with the meal for three days. The cinchophen used was the "pure," or snow white cinchophen prepared by the Calco Company, and was completely soluble in alkaline aqueous solution and devoid of odor; it is less toxic and a different product than that yellowish white product marketed several years ago.[12] The determinations made during the control period were repeated during the test period. However, cinchophen excretion in the bile every twenty-four hours was determined by the method of Bradley.[2] The diet consisted of a commercial dog food (Pard) which was supplemented with dried yeast and cod-liver oil, the dogs consuming the same amount of the diet daily. (B) The second type of experiment consisted in giving cinchophen as above, except it was given for a period of fourteen days. (C) The third type of experiment consisted of giving cinchophen and then returning all bile formed every hour. The detailed method will be given later.

TABLE I

THE EFFECT OF 1 GM. OF CINCHOPHEN DAILY FOR THREE DAYS ON CHRONIC BILIARY FISTULA DOGS, NO BILE OR BILE SALTS BEING GIVEN

DOG NO.	WT. (KG.)	VOLUME (C.C./24 HR.)			CHOLIC ACID (MG./24 HR.)			PIGMENT (MG./24 HR.)			CHOLESTEROL (MG./24 HR.)			RECOVERY OF CINCHOPHEN	
		C	T	% CHG.	C	T	% CHG.	C	T	% CHG.	C	T	% CHG.	TOTAL OUTPUT (GM.)	% REC.
2	10.0	131	266	+103	901	726	-19	88	77	-13	12	16	+ 33		
		137	260	+ 90	1,241	1,268	+ 2	123	124	0	12	18	+ 50	2.25	75
3	11.0	130	282	+117	1,472	1,136	- 9	150	120	-20	13	22	+ 69		
		143	287	+100	1,518	1,516	0	133	140	+ 5	15	23	+ 53	2.11	70
4	8.4	122	287	+135	1,346	1,441	+ 7	109	133	+22	12	18	+ 50		
		123	280	+127	1,337	1,561	+16	101	151	+50	13	22	+ 69	1.88	63
5	11.1	140	248	+ 77	1,139	1,175	+ 3	103	126	+22	16	35	+118		
		132	282	+113	1,218	1,257	+ 3	100	138	+38	16	34	+112	1.84	61
6	12.0	130	263	+102	1,420	1,226	-13	85	138	+62	12	25	+108		
		130	277	+113	1,421	1,422	0	120	160	+33	18	43	+138	1.98	66
Average 5 dogs —10 tests		132	273	+106	1,301	1,030	0	111	131	+18	14	26	+ 85		
3 Gm. ketochol		126	251	+ 99	1,468	1,568	+ 6	114	107	- 7	11	14	+ 27	0.728	24
3 Gm. decholin		128	264	+106	1,473	1,647	+12	114	129	+13	11	8	- 27	0.835	28

RESULTS

A. The Effect of Cinchophen Administration for Three Days.—On the average, 1 Gm. of cinchophen daily, given as two doses of 0.5 Gm. with the morning and evening meal, increased the volume output of bile over the control by 106 per cent (Table I). The range of increase was from +77 to +136 per cent above the control output. The bile was clear and odorless, and at no time resembled the bile obtained in the presence of an infectious hepatitis. The choleresis pro-

duced was analogous to that obtained with 3 Gm. of dehydrocholanic acid salts (ketochol) or of sodium dehydrocholate (decholin, sodium).[7]

On the average *cholic acid synthesis* was not changed, although the individual tests show a range of from –19 to +16 per cent. A plus or minus range of 10 to 15 per cent occurs in some groups of dogs, however, under apparently constant environmental conditions. The *total pigment output* showed a +18 per cent increase on the average; it was increased or not altered in all but three tests, which is not a significant increase according to our previous observations[7, 8] on this type of biliary fistula preparation, in which a variation of + or –25 per cent of the mean may occur. A definite increase in *cholesterol output* occurred. The output of cholesterol, however, is increased when a choleresis is produced by the administration of conjugated ox bile acids or the return of dog's bile. According to our observations,[7] the cholesterol output varies + or –20 per cent under constant environmental conditions.

From 61 to 75 per cent of the cinchophen administered was recovered in the twenty-four-hour volume of bile. This agrees with the observations of Bradley and Ivy,[2] who recovered from 59 to 78 per cent in semiacute biliary fistula dogs. The test for cinchophen was negative in the precontrol and postcontrol cinchophen period.

Comment: It is clear from the results that cinchophen administered in 0.5 Gm. doses with the meals to the 5 dogs used produced no evidence of toxicity, using volume output, cholic acid synthesis, pigment, and cholesterol output as criteria. It is possible, however, that a period of three days of administration is not sufficiently long to produce hepatotoxic signs.

B. The Effect of Cinchophen Administration for Fourteen Days.—Following the same procedure as in the experiment above, 1 Gm. of cinchophen was given daily for fourteen days.

Dog 5 had suffered a partial obstruction of the bile duct several days prior to the start of the control period. We decided to use the animal to ascertain what might happen when cinchophen is administered during a time that the liver is recovering from an obstruction. During the obstruction the bile had a bad odor and was infected. This tended to clear when the obstruction was relieved.

TABLE II

THE EFFECT OF 1 GM. OF CINCHOPHEN DAILY FOR FOURTEEN CONSECUTIVE DAYS ON CHRONIC BILIARY FISTULA DOGS, NO BILE OR BILE SALT BEING GIVEN

DOG NO.	WT. (KG.)	VOLUME (C.C./24 HR.)			CHOLIC ACID (MG./24 HR.)			CHOLESTEROL (MG./24 HR.)			PIGMENT (MG./24 HR.)		
		C	T	% CHG.	C	T	% CHG.	C	T	% CHG.	C	T	% CHG.
2	10.0	129	232	+ 80	1,184	1,152	- 2	14	20	+42	114	136	+ 9
2'	11.0	129	291	+125	1,380	1,175	-14	13	18	+39	107	139	+30
83	9.0	99	230	+132	1,455	1,601	+10	16	23	+44	126	120	- 4
4	8.4	127	322	+154	1,468	1,541	+ 5	16	29	+81	157	200	+27
5*	11.1	134	255	+ 90	1,106	718	-35	18	27	+50	111	122	+ 9
83†	9.0	94	219	+133	1,199	1,289	+ 7	16	19	+18	128	143	+11
Average		119	258	+116	1,295	1,246	- 3	15	23	+50	124	127	+ 2

*See Table III.

†Cinchophen given for thirty-three days.

The results obtained from six experiments on the 5 dogs are shown in Table II. The results are approximately the same as those obtained in the three-day experiment.

In Dog S3, the second test on the dog recorded in Table II, cinchophen was administered for thirty-three days. During this period the animal consumed all his diet with appetite, though no bile was returned or bile salts were administered. The animals as well as the composition of the bile remained normal. The average volume output during the last eleven days was 254 c.c. per day, and the average cholic acid synthesis was 1,336 mg; both tended to be higher than during the earlier part of the experiment. It should be remarked that other dogs may synthesize and excrete less cholic acid during the first than during the second week.

TABLE III

THE EFFECT OF 1 GM. OF CINCHOPHEN DAILY IN THE PRESENCE OF LIVER DISEASE*

DOG. NO.	WT. (KG.)	DATE	POSTOP. DAY	HR. FLOW	DIET	VOLUME (C.C.)	CHOLIC ACID (MG.)	CHOLESTEROL (MG.)	PIGMENT (MG.)
5	11.1	12/28/40	101	24	Reg.	214	421		99.1
		12/29/40	102	24	Reg.	223	580		103.2
		12/30/40	103	24	Reg.	247	370	16.3	134.1
		12/31/40	104	24	Reg.	249	598		148.1
		1/ 1/41	105	24	Reg.	261	548		157.9
		1/ 2/41	106	24	Reg.	258	564		118.7
		1/ 3/41	107	24	Reg.	265	815		116.7
		1/ 4/41	108	24	Reg.	262	838	38.2	161.1
		1/ 5/41	109	24	Reg.	259	958		170.4
		1/ 6/41	110	24	Reg.	277	865		101.4
		1/ 7/41	111	24	Reg.	286	972	22.6	123.3
		1/ 8/41	112	24	Reg.	238	857		94.0
		1/ 9/41	113	24	Reg.	260	832		88.6
		1/10/41	114	24	Reg.	277	837	29.9	87.2
Average						255	718	27.0	121.7

*An obstruction of the bile duct was relieved three days before the start of this experiment. The low cholic acid output during the first few days is attributable to an hepatitis. The animal consumed all the diet each day.

No bile was returned; no bile salts were given.

The results on Dog 5 are of particular interest because the dog had not fully recovered from a partial biliary obstruction and hepatitis when cinchophen medication was started. The daily determinations are recorded in Table III. At the start of the experiment only 0.3 to 0.4 Gm. of cholic acid was synthesized daily. Later, cholic acid output increased to 0.8 to 0.9 Gm. daily.

Comment: On the basis of the criteria used, the administration of 1 Gm. of cinchophen daily, 0.5 Gm. with each meal, for fourteen days and in one case for thirty-three days caused no evidence of hepatic injury.

In the dog recovered from partial obstruction and hepatitis, the liver manifested a gradual recovery. Whether the recovery of the animal was retarded cannot be stated, since such animals in our experience may recover in from three to fourteen days when no cinchophen is given. However, in such instances we always give a bile salt preparation or return bile with the idea of flushing the ducts, since obstruction, as a rule, occurs in our dogs only when they are kept on food without bile salts or the return of bile. The thick bile formed in the absence of the return of bile or bile salts definitely favors obstruction and infection

of the bile; this is the chief reason why we never deprive our dogs of bile for a longer period than from five to seven days, except when we give them some other choleretic.

The fact that no cinchophen could be found in the bile twenty-four hours after the last dose was given shows that it is rather rapidly excreted or metabolized. The fact that we were able to obtain from 61 to 75 per cent of the administered cinchophen in the bile shows that cinchophen in the presence of an intact biliary tract may undergo repeated enterohepatic circulation. Since in our dogs the cinchophen given could undergo only one enterohepatic circulation, *it is possible that hepatic injury may occur in the intact animal due to repeated enterohepatic circulation.* This hypothesis was tested by the following experiment.

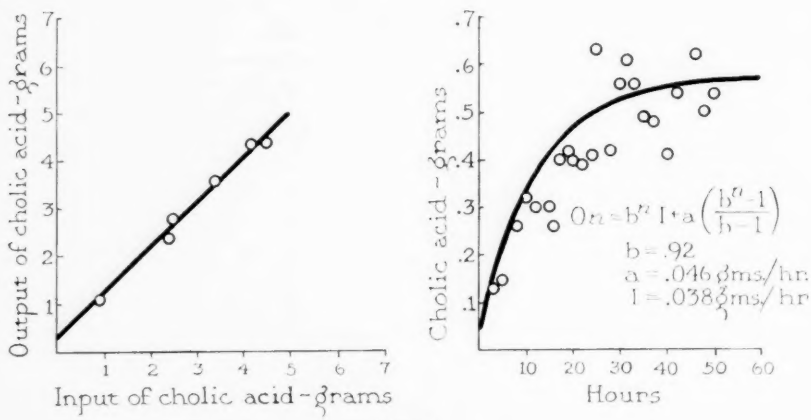

Fig. 1.

C. *The Effect of Repeated Enterohepatic Circulation of Cinchophen in the Dog.—Method.* One biliary-duodenal fistula "suction" dog[9] was used. The dog weighed 9.0 kg. The bile was collected in a glass cylinder and could be returned directly into the intestine through the duodenal fistula. The animal was fed at eight-hour intervals one-third of his daily ration of the standard diet. The bile was collected and returned on an hourly schedule, the volume being recorded and a 0.1 c.c. sample being retained for cholic acid assay. The bile collected hourly was returned to the intestine during a five- to ten-minute period for forty-eight hours. After a three-day control period of no return of bile the hourly return was started and 333 mg. of cinchophen was mixed with each eight-hour feeding.

Results. It has been shown previously[10, 11] that when bile is returned to an animal every hour after a period during which no bile has been returned, the cholic acid output, due to enterohepatic circulation, will increase according to a definite curve or formula and will in time reach a relatively constant or homeostatic level, at which synthesis is balanced by the "destruction" of cholic acid. In view of this fact, it was thought that if cinchophen disturbed cholic acid synthesis, the disturbance would result in a modification of the "normal" curve of accumulation. Therefore, the data obtained on cholic acid output on the hourly return of bile with cinchophen administration was treated mathematically as in previous work.[10, 11]

When the input of cholic acid is plotted against output of cholic acid, a linear relationship occurs (Fig. 1). When the results are analyzed mathematically and the equation of the straight line, output = "b" times input plus "a," was solved, it was found that "b," the slope of the line or per cent of recovery was 92 per cent, and "a," the point of intercept on the ordinate, or basal cholic acid output on the diet, was 0.046 Gm. per hour. The curve that fitted this straight line was predicted by the following equation: $On = b^nI + a\left(\frac{b^n - 1}{b - 1}\right)$, where "On" is the output of cholic acid at any subsequent hourly period, and "I" is the actual initial dose of cholic acid administered at the first return of bile. The curve predicted by this equation, which has been tested and established by previous experimental results,[10, 11] is shown in Fig. 1. The predicted homeostatic level was 0.60 Gm. per hour and that actually obtained experimentally was 0.55 Gm. per hour. The predicted cholic acid synthesis per hour was 0.046; the actual synthesis found was 0.050 Gm. The predicted recovery was 92 per cent, the actual was 91 per cent. The actual curve of output (Fig. 1) approximates fairly closely the predicted curve, though it is somewhat lower at the first, which is, in general, true in other such tests when cinchophen is not given.

Comment: The results show definitely, insofar as this dog is concerned, that repeated enterohepatic circulation of cinchophen during a forty-eight-hour period causes no decrease in cholic acid output.

DISCUSSION

The effect of cinchophen feeding on the secretion of bile has been studied under various conditions: (a) when fed for three-day periods; (b) when fed over a period of fourteen consecutive days, and thirty-three days in one animal; and (c) when making 24 enterohepatic circulations a day for two days. In no case were we able to show that cinchophen acted like a hepatotoxin as determined by bile analyses. The cholic acid output, which we believe is the most sensitive indicator of liver dysfunction, was not altered significantly. This was especially true of the average results on a number of biliary fistula dogs, although individual tests may show a greater variability in cholate synthesis. In all cases due to choleresis the cholic acid concentration in the bile was decreased, but the total cholate output remained on the average the same as the control. Similar results were obtained using potent oxidized bile acid choleretics.[7] It was also shown that cinchophen does not affect the regulation of cholic acid output by the liver. When the bile, containing cinchophen, was returned every hour during the day, the cholate output gradually increased and reached the homeostatic level predicted by a methematical equation.

We have no explanation for the increased cholesterol output due to cinchophen feeding; this occurs whenever a choleresis is produced by any procedure known. Since the cholic acid output was not simultaneously increased, the bile acid: cholesterol ratio would be decreased, which *may* tend to predispose to a precipitation of fatty acid and cholesterol in the gall bladder in the presence of a condition favoring the diffusion of bile salts from the organ.

These experiments establish cinchophen as a potent hydrocholeretic even as effective as dehydrocholic acid (decholin) or mixed ketocholanates (ketochol).

When 3 Gm. of these bile acids were administered per day, a +103 per cent increase, on the average, in volume output was obtained. One gram of cinchophen daily, whether administered over a short or long period of time, gave an average increase of +106 per cent. Comparing these substances on a gram-weight basis, cinchophen is three times more effective than dehydrocholic acid as a hydrocholeretic. The reason for this marked choleresis is not definitely known. In acute and chronic biliary fistula dogs the choleresis produced by cinchophen was associated with the excretion of 60 to 75 per cent of the administered drug. When 10 to 50 mg. of cinchophen were injected intravenously into acute and chronic biliary fistula rabbits, no hydrocholeresis occurred, and only very small amounts of cinchophen were excreted in the bile.[4] These results indicate that in the dog, but not in the rabbit, the liver is especially concerned in the excretion of cinchophen and in the process of its excretion water is excreted with the drug. It is quite probable that the ease of solubility of cinchophen in bile and its osmotic activity therein are important factors contributing to the hydrocholeresis produced by cinchophen administration in the dog.

SUMMARY AND CONCLUSIONS

One gram of "pure" cinchophen (Calco Company) was fed daily (0.5 Gm. with each meal) to 5 chronic biliary fistula dogs for three- and fourteen-day periods, and in one instance for thirty-three days, and the effect on bile volume, cholic acid, total pigment, and cholesterol outputs was determined. The amount of cinchophen recovered in the bile was also determined. It was found that this daily dose of cinchophen caused a marked hydrocholeresis associated with no significant change in cholic acid and total pigment outputs, a marked increase in cholesterol output, and a recovery of 60 to 75 per cent of the administered cinchophen.

On a gram-weight basis cinchophen is three times as effective as a hydrocholeretic as the most potent bile acid choleretic, namely, dehydrocholic acid.

When bile, formed under the stimulus of cinchophen feeding, is returned every hour during the day for two days to simulate conditions in the intact animal and to cause repeated enterohepatic circulation of the cinchophen and bile salts, the cholic acid regulatory mechanism was not disturbed and the cholate output increased and closely approximated the predicted homeostatic level of the nonmedicated animal.

In the presence of an already injured liver, cinchophen feeding did not definitely increase the severity of the hepatitis; it cannot be said that the cinchophen aided recovery, though recovery occurred during cinchophen medication.

Therefore, from our studies on chronic biliary fistula dogs, cinchophen, when fed in 1 Gm. daily doses, does not seem to act as a hepatotoxin on the basis of the criteria used. To what extent the "margin of safety" in the liver was affected by the cinchophen is not clearly determined by the type of experiment used in these studies.

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DRUG PROPHYLAXIS AGAINST LETHAL EFFECTS OF SEVERE ANOXIA

IV. A Standardized Technique
Influence of Body Weight, Injection of Saline, Muscular Restraint, Rate of Ascent, and Pretreatment With Oxygen or Helium-Oxygen in Anoxic Mice

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THE influence of several medicinal agents upon resistance to acute anoxia has been studied by numerous investigators, and their reports are summarized in the first paper of this series.[1] Major variations of technique and the lack of critical examination of the findings of different workers may be responsible for discordant conclusions regarding individual agents. It is difficult to make any adequate correlation of prophylactic effects of different agents when results obtained under widely different experimental conditions are compared. Relative efficacy of different agents may be estimated accurately only through the use of one or more standard techniques, and a concept of the relative involvement of the various physiologic processes known to be concerned in the lethal action of anoxia may then be gained.

Factors of simplicity, inexpensiveness, and uniform reproducibility are of primary importance in a test of prophylactic effects against anoxia, and these considerations governed the development of the present method. Since a standard technique should involve a test of effects of agents in intact mammals, with simultaneous observation of lethal effects of anoxia in sufficient numbers of treated and untreated animals to permit statistical evaluation of results, mice are best suited for use in the proposed method.

The most easily controlled type of anoxia is anoxic anoxia, as produced by exposure to low atmospheric pressures. In contrast, controlled degrees of anemic, histotoxic, or stagnant anoxia are extremely difficult, if not impossible, to obtain,

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especially in large groups of animals. Further, anoxic anoxia is probably more commonly encountered in diseases of man than are the other types. It may be emphasized, however, that results of tests upon small mammals and upon lethal effects of acute exposure to simulated high altitudes necessarily have no immediate applicability to the treatment of the moderate degrees of anoxia occurring in many diseases of man or in modern aviation practice. The function of a simple test of the antagonistic action of different agents toward lethal effects of anoxia in animals is one of orientation, after which more intensive study may suggest the possible clinical usefulness of those agents which show promise in examination by more elaborate techniques. In some instances, agents which are not prophylactic against, or are even synergistic with the lethal effects of exposure to simulated high altitudes may, nevertheless, be useful in the treatment of effects of moderate degrees of anoxia. Recognition of the limitations of such agents in the treatment of severe anoxia is still necessary for full knowledge of their action.

The scope of the problem concerning various agents other than oxygen, which may have protective value in severe anoxia, has been presented elsewhere.[1] The prophylactic effects of certain convulsants,[1] narcotics,[2] and of the optical isomers of benzedrine[3] have been studied by the technique described below.

METHOD

The method involves the use of sufficiently large numbers of mice for statistical significance, simultaneous exposure of an untreated control group of equal size to the same conditions of anoxia, and a comparison by statistical criteria[4] of mortality in control and treated groups at the time approximately 50 per cent of the control mice have died from anoxia.

Young healthy adult mice of the same strain are weighed to the nearest gram and are treated intraperitoneally with the test agent dissolved in saline. Control mice are treated with an equal volume of saline at the same time. This step is unnecessary under ordinary conditions but is desirable since it makes the degree of hydration more uniform in all mice. The interval between treatment and exosure to anoxia may vary with different agents according to their time of maximum action, but, in general, should be at least one hour for the longer-acting agents to minimize effects of the difference in time of treatment of the first and last mice injected.

Exposure to anoxia is produced in a cylindrical decompression chamber resembling that of Kolls and Loevenhart.[5] This tank is constructed of ¼ inch boiler plate, oxyacetylene-welded at all seams. Its horizontal diameter is 90 cm. and its height 45 cm. The capacity is approximately 285 liter. The tank is provided with three double-strength plate glass windows, reinforced with metal bars, to permit illumination and observation; a U-shaped mercury manometer with one arm open to the outside atmosphere; two adjustable inlet valves set oppositely at the top; and an outlet connected with a vacuum pump of high capacity. Ventilation is adequate in all parts of the tank at the lowest pressures used with biologic material, and there is no possibility of accumulation of carbon dioxide under the conditions of the proposed test.

Although it is recognized that no valid single degree or even moderately narrow range of anoxia can be taken as the point at which 50 per cent of control mice die,[1] the conditions of producing anoxia should be kept rigorously constant. The varying resistance of different control groups to anoxia then probably reflects constitutional differences, for there is no correlation with the pre-experimental barometric pressure, humidity, or apparent physiologic condition of the mice. This variation in tolerance on different days necessitates the simultaneous exposure of treated and control groups to identical conditions.

Mice within the tank are rapidly exposed to a reduced pressure of about 523 mm. Hg, which simulates an altitude of approximately 10,000 feet. This pressure is maintained for ten minutes. After this period of equilibration of blood and tissue gases with this level of reduced pressure, the pressure is further reduced at a rate corresponding to an ascent of 1,000 feet per minute. Mice usually tolerate this progressive anoxia until a reduced pressure of less than 226 mm. Hg is attained within some thirty minutes after beginning exposure; this pressure corresponds to that at an altitude of 30,000 feet. Approximately 50 per cent of control mice die at the time atmospheric pressures of 170 to 141 mm. Hg are attained, which correspond to simulated altitudes of 36,000 to 40,000 feet.

Termination of exposure is governed by mortality of 50 per cent of the control mice rather than by attainment of a fixed level of anoxia. Mortality of 50 per cent of the control group affords the most significant point for statistical comparison of mortality in treated groups and estimation of either protective or harmful effects of the different agents. An ideal prophylactic would completely prevent lethal effects of anoxia at a level causing 100 per cent mortality of control mice. Except oxygen, no agent has yet been found capable of this.

After exposure, mice are observed for an additional forty-eight hours to include any possible late effects of treatment. Latent lethal effects have not been encountered with the agents tested thus far. Necropsies are occasionally made of mice dying during exposure to anoxia. Groups of mice showing pneumonitis should not be used.

The influence of diet, and especially of various hypervitaminoses, is to be studied in detail. As the test has been used to date, mice have been supplied with a standard diet and water, available at all times. The mice have been subjected to anoxia in the early afternoon. Since mice are chiefly nocturnal feeders, the stomach and small intestine are usually fairly empty at this time and the mice are in a postabsorptive state.

BODY WEIGHT

Immature animals are less susceptible to lethal effects of anoxia than are adults,[6, 7] and smaller species of animals are less susceptible to aeroembolism than are larger species.[8] In a test of tolerance to anoxia the validity of results obtained with unselected groups of young adult mice may be questioned. If the smaller mice of such groups are significantly less susceptible to anoxia, and if the numbers of smaller mice in control and treated groups do not coincide, errors might arise from this cause. Data have accumulated from previous studies which bear upon this problem.

The mean body weight of 160 mice dying during exposure for thirty minutes to severe anoxia was 16.8 ± 0.21 Gm., while the mean body weight of 240 mice surviving the same conditions of anoxia was 16.1 ± 0.17 Gm. The difference between these means is 0.7 ± 0.27, signifying[9] a p of <0.01. However, this difference is more apparent than real, for the data were integrated from results with several groups of mice of different mean body weights and of differing tolerance to anoxia, as noted by differences in mortality ratios in the individual experiments. The statistical treatment of such data cannot be considered conclusive. With smaller groups[2] simultaneously exposed and, therefore better controlled, the differences between mean body weights of mice surviving and those dying under identical conditions of anoxia were not significant, although the mice dying during anoxia were uniformly slightly heavier than those simultaneously surviving.

Of 100 mice from the same colony, 52 survived and 48 died when subjected simultaneously to severe anoxia produced according to the proposed technique. Mean body weights of these two groups were 15.6 ± 0.47 and 16.0 ± 0.40 Gm., respectively. The difference between these means is not significant, although again the survivors were slightly lighter than those dying in anoxia.

Neonatal tolerance of the rat[7] to anoxia is rapidly lost within eighteen days of postnatal life. It does not appear to be necessary that young adult mice should be carefully selected within a narrow weight range for use in tests of tolerance to anoxia. It may be noted that even if the coefficient of correlation of body weight and tolerance to anoxia were a significantly large negative value, the source of error from this cause in the proposed technique would still be dependent upon inequalities in numbers of small mice in control and treated groups. The use of large groups of mice minimizes this hazard.

INJECTION OF SALINE

It was previously reported[1] that intraperitoneal injection of 50 c.c. per kilogram of physiologic saline fifteen minutes before exposure to anoxia has no harmful effect on tolerance of mice. Thirteen of 20 mice so treated died under anoxic conditions simultaneously causing death of 14 of 20 untreated mice. Since then mice used as controls have been treated routinely with saline injections in amounts as great as 60 c.c. per kilogram giving one hour before exposure, without apparent effect on tolerance. If appreciable hydremia is produced by these massive doses of saline, the resultant anemia from dilution of the blood is insufficient to affect tolerance to anoxia. If blood volume is actually increased by these saline injections, an undesirable accentuation of the cardiac dilatation known to occur in anoxia[10] would probably outweigh any possible beneficial effects. Whatever the circulatory effects of massive doses of saline may be, however, they do not appreciably affect the lethal action of severe anoxia under the conditions of the proposed test.

MUSCULAR RESTRAINT

Mice become hyperactive during induction of anoxia, more or less in proportion to the rate of production of anoxia. In susceptible mice, and in the majority after administration of subconvulsive doses of central nervous system stimulants,[1]

this hyperactivity leads to severe clonic or epileptiform convulsions. Mice exhibiting these convulsions usually become comatose and die within a short period. Typical tetanic convulsions are infrequent in anoxic mice, and the cause of the usual convulsion in anoxia is probably cortical and sympathetic stimulation, or depression of the motor inhibitory mechanisms, rather than nervous effects of the alkalosis due to hyperpnea in early anoxia. The possibility that the hyperactivity is a response to painful stimuli from aeroemboli in joints or other tissues is negated by findings which indicate that aeroembolism is not an appreciable factor under the experimental conditions. Distention of hollow viscera due to expansion of contained gas under reduced pressure could, however, give rise to strong pain stimuli, and neuralgic pain arising from local effects on nerve trunks may occur in mice as in man.[8]

The metabolic rate is much higher during active exercise than in quiescence, and tolerance to anoxia should thereby be decreased. A significant increase in tolerance to anoxia is shown by mice fully narcotized with certain depressants[2] over that of control mice with normal activity. In order to test whether simple physical restraint would produce like results, the following two experiments were made.

Ten mice were confined, each within a 60 c.c. beaker covered with a single layer of cotton gauze. Under anoxic conditions in which 18 of 40 normal mice died when simultaneously exposed and allowed to run about inside the decompression chamber, all the 10 mice confined in the beakers died. It was felt that perhaps adequate ventilation was prevented and also that the confinement was not sufficient to restrain the mice from struggling, although none showed frank convulsions.

Twenty mice were therefore confined, each within a tube of light 14-mesh wire gauze. The tubes were made of a single thickness of the wire gauze, closed by pinching the ends and loosely stitching the longitudinal seam with light copper wire. Each tube was constructed to fit the occupant snugly but to prevent major movements of the body and full extension of the limbs. The mice were free to move easily forward along the tube but could not turn around. The usual response to confinement was sleep. Special care was taken not to make the tube too small for full respiratory movements, and the longitudinal closure was loose enough to permit a considerable amount of enlargement of the lumen with little resistance, to correct for any moderate increase in body volume at reduced pressures.

Of the 20 mice thus restrained, all died under conditions of anoxia in which only 9 of 20 control mice died when allowed the freedom of the decompression chamber.

In both experiments the rate of production of anoxia conformed to that noted in the proposed method, with a plateau for ten minutes at a simulated altitude of 10,000 feet, followed by a simulated rate of ascent of 1,000 feet per minute to 41,000 feet. With this relatively slow rate of ascent, mice show a minimal amount of excitement, although convulsions commonly precede death at the higher levels of anoxia. It may be concluded that, in testing prophylactic drugs by the proposed technique, close confinement of the experimental animals is neither beneficial nor desirable and does not resolve irregularities in oxygen

consumption due to differing activities of the mice as efficiently as does the use of a rate of production of anoxia which minimizes excitement. The harmful effects of restraint may be due to marked isometric muscular activity resulting in an increase in oxygen consumption actually greater than that in mice allowed to run about at will, and perhaps also to increased excitement and sympathico-adrenal stimulation consequent to confinement.

RATE OF ASCENT

Tolerance to anoxia is related to the rate of production of anoxia in two major ways: through development of compensatory mechanisms of the organism and through cumulative effects of prolonged anoxia. When animals are very rapidly exposed to low atmospheric pressures, limitations of the rate at which the body can lose oxygen and the possibility of occurrence of aeroembolism are also involved. Heim and Armstrong[11] have reported an increase of about 50 per cent in altitude tolerance as the rate of ascent is increased from 100 to 30,000 feet per minute. They believe that effects of anoxia upon the central nervous system are greatest with rapid production of anoxia, and that cardiovascular effects are greatest with slow ascents. It would follow that different types of drugs might exhibit different degrees of prophylaxis according to the rate of production of anoxia. Tests of lethal effects by different techniques of producing anoxic anoxia are of interest as an index of the possibility of aeroembolism. Dilution techniques[12] completely avoid any possibility of aeroemoblism but present certain technical difficulties. Anoxia was, therefore, produced in the decompression chamber at five different simulated rates of ascent and lethal effects of these different treatments were observed.

Five groups of 20 mice each were subjected to anoxia produced at one of the rates noted in Fig. 1. Curve A represents a simulated rate of ascent of approximately 15,000 feet per minute until an approximate altitude of 37,000 feet was attained, followed by a slow ascent of about 100 feet per minute for ten minutes. Approximately one-half of the mice died at a simulated altitude of 37,500 feet, six minutes after beginning exposure to anoxia. Curve B represents a simulated rate of ascent of about 1,000 feet per minute. One-half of the mice died at a pressure corresponding to 42,000 feet, thirty-eight minutes after beginning exposure to anoxia. Curves C and D represent rates of ascent coinciding with that of curve A until approximate altitudes of 11,000 and 21,500 feet, respectively, were attained. These levels of anoxia were then maintained for ten minutes, after which a further simulated rate of ascent of approximately 2,000 feet per minute was begun. One-half of the mice so treated died at a simulated altitude of 37,000 feet twenty-three minutes after beginning exposure, and at a simulated altitude of 42,000 feet twenty minutes after beginning exposure, for treatments represented by curves C and D, respectively. Curve E represents rapid ascent to about 32,000 feet, maintenance of this level for ten minutes, and a further ascent at the rate of about 1,000 feet per minute. One-half of the mice treated in this way died at an approximate altitude of 32,000 feet, nine minutes after beginning exposure to anoxia.

Curves A, D, and E terminate at the level at which the last animal of the group died. Two of the 20 mice subjected to the treatment represented by

curve C recovered after exposure, despite brief apnea in both. Seven of the 20 mice subjected to the slow rate of ascent represented by curve B recovered after exposure. Comparison of the minimal pressures attained in the different treatments is meaningless, since these pressures reflect the tolerance of only the most resistant mice in each group. A more significant comparison is obtained by consideration of the levels of anoxia at which 50 per cent of the mice died.

The above tests were made consecutively, under identical conditions of barometric pressure, humidity, and temperature, and all mice were of the same strain with identical pre-experimental care and diet. The results indicate that aeroembolism is not a significant factor as a cause of death under the conditions of the proposed test. After a return of mice to room conditions, even those subjected to the most severe conditions noted in Fig. 1 showed no gaseous emboli in vessels of the pia mater or in the abdomen.

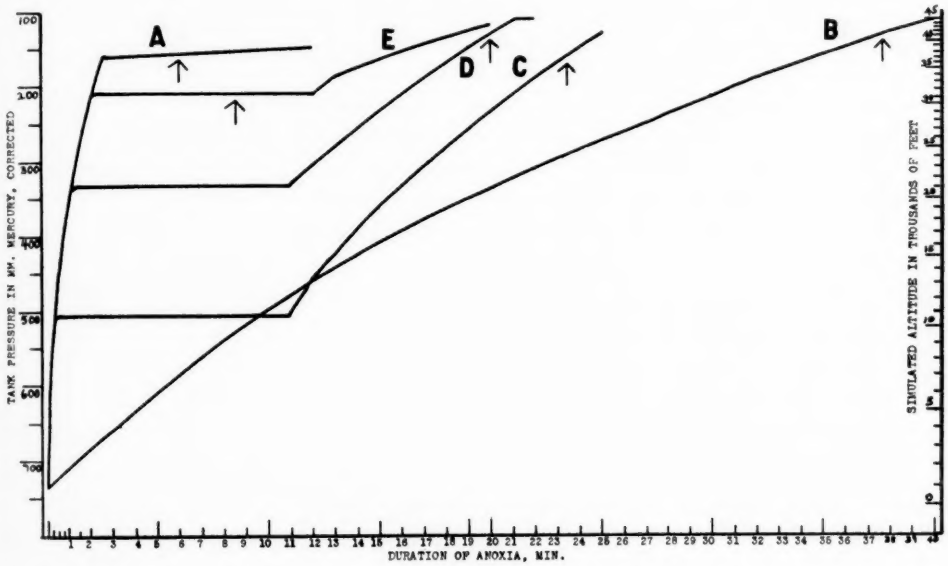

Fig. 1.—Effect of simulated rate of ascent on tolerance of mice toward lethal effects of anoxia ↑ denotes point at which one-half of the mice died.

The results also indicate that a simulated rate of ascent of 1,000 feet per minute is slow enough to permit various compensatory changes in mice but is rapid enough to avoid any marked cumulative effects of anoxia.

PRETREATMENT WITH OXYGEN

Thirty mice were placed in an 8 liter sealed glass chamber containing 1 kg. of soda lime so placed that the mice did not have access to it. Oxygen was passed through the chamber at a rate of 2 liters per minute for ninety minutes. The mice were then placed in the decompression chamber together with 30 mice which had been kept under normal atmospheric conditions. The pressure was quickly reduced to a simulated altitude of 20,000 feet and was held at this level for ten minutes. The pressure was then further reduced at a rate simulating an ascent of 2,000 feet per minute until an altitude of about 38,000 feet was attained, and this degree of anoxia was then maintained. The total time of exposure was thirty minutes.

Twenty-one of the 30 previously untreated mice died from anoxia, while only 14 of the 30 mice which received oxygen prior to exposure died. Although this difference in mortality is large, it cannot be considered as a statistically significant difference since its p is greater than 0.05.

The rate at which oxygen was supplied during the pretreatment period is equivalent to a complete change of the atmosphere within the chamber every 3 to 3.5 minutes, or a total of 26 to 30 complete changes in ninety minutes. Under these conditions appreciable denitrogenation of the tissues occurs. The significance of this in regard to aeroembolism is discussed below. Consideration of the factors involved in this experiment led to observation of effects of pretreatment with helium-oxygen.

PRETREATMENT WITH HELIUM-OXYGEN

Thirty mice were treated in the same way as in the pretreatment with oxygen alone, except that pure oxygen was passed through the chamber at the rate of 2 liters per minute for only thirty minutes. The chamber was then rapidly flushed with 18 liters of helium, quickly followed by 30 liters of a mixture of 80 per cent helium and 20 per cent oxygen. The outlet tube from the chamber was closed and the chamber was connected with a reservoir of pure oxygen maintained at atmospheric pressure, thus permitting the flow of oxygen at the rate at which the soda lime within the chamber absorbed carbon dioxide excreted by the mice. Mice were kept in this atmosphere of 80 per cent helium and 20 per cent oxygen for one hour, during which period the chamber was flushed three times with 80 per cent helium and 20 per cent oxygen in order to remove any accumulated nitrogen.

The mice were then subjected to anoxia in the decompression chamber, together with 30 previously untreated mice. The procedure of producing anoxia was the same as with mice pretreated with oxygen alone.

Of the 30 control mice, 11 died during the exposure to anoxia while only 6 of the 30 mice pretreated with helium-oxygen died. Again this difference in mortality is large, but it is not statistically significant. The implications of these data are discussed below.

DISCUSSION

Factors which may influence results in the proposed test have been mentioned, and their significance has been appraised experimentally. Body weight of young adult mice is not correlated significantly with resistance to anoxia. Therefore, groups of young adult mice need not be carefully selected within a narrow weight range for use in the test. Massive intraperitoneal injections of saline have little effect on tolerance to anoxia, so that control groups need not receive amounts of saline equivalent to those given as a solvent for the test agents, although this treatment with placebo injections in control mice is probably desirable to render the degree of hydration more uniform in all mice. Muscular restraint, as produced by two methods, fails to increase tolerance of mice to simulated high altitudes and appears markedly detrimental, at least under conditions of decompression which minimize excitement in mice allowed the freedom of the chamber.

The remaining factors involve the important question of the possibility of occurrence of aeroembolism in mice subjected to the proposed experimental technique. If an appreciable number of deaths under the experimental conditions can be ascribed to aeroembolism rather than to anoxia, these conditions obviously cannot yield a significant measure of prophylactic actions of drugs against lethal effects of anoxia.

Effects of decreased atmospheric pressures upon the blood and spinal fluid have been studied intensively.[8, 13] Armstrong[8] has pointed out that in man an acute exposure to decreased atmospheric pressure, such as might occur in sudden failure of a sealed cabin in aircraft at high altitudes, would result in anoxia at altitudes below 30,000 feet, in aeroembolism at altitudes between 30,000 and 63,000 feet, and in rapid vaporization of body fluids at altitudes above 63,000 feet. In experimental observations, however, he[8] found that while goats may show the presence of nitrogen bubbles in blood during exposure to high altitudes, this phenomenon is not apparent in rabbits subjected to a simulated rate of ascent of 40,000 feet in forty seconds. With goats nitrogen bubbles appear in the spinal fluid at a simulated altitude of 18,000 feet, and increase as ascent is continued. Intracranial pressure simultaneously increases.

Walsh and Boothby[13] investigated effects of low atmospheric pressure on spinal fluid in man and found that exposure to simulated altitudes as low as 10,000 to 12,000 feet elicits the appearance of fine bubbles "like those arising from champagne" and that these increase in size and number until an altitude of 28,000 feet is attained. On maintenance of this level for five minutes, the bubbles disappear. Intraspinal pressure increases progressively by some 3 cm. after the appearance of bubbles. Similar findings were observed in another subject partially denitrogenated by prior exposure to an atmosphere of pure oxygen. In vitro tests substantiated these results. With a monkey, however, a roentgenogram did not disclose the presence of any accumulation of gas in the ventricles of the brain even after a rapid ascent to a simulated altitude of 40,000 feet.

From these reports it may be concluded that smaller mammals withstand lower levels of atmospheric pressure before exhibiting formation of nitrogen bubbles in blood and spinal fluid. This characteristic is probably related to different rates of elimination of nitrogen due to differences in circulatory rates. Thus, in an atmosphere of pure oxygen, 95 per cent denitrogenation occurs within two hours in dogs and four hours in man, and the circulatory rate of the dog is about twice that of man, expressed in terms of blood supply per kilogram of body weight.[14] Extrapolation of results with rabbits and monkeys to mice appears justifiable, and the possibility of occurrence of aeroembolism in mice under the conditions of the proposed test would seem remote. Three points of evidence in the present study support this view and suggest that there is no hazard of aeroembolism under the experimental conditions. A further, though minor, confirmatory point involves the lack of a significantly demonstrable difference in tolerance in adult mice of different body weight; although there may be no valid relative difference in amount of fatty tissue in large and small mice, it is reasonable to assume a difference in absolute amount of body fat and fatty tissue acts as a reservoir for nitrogen, favoring aeroembolism.[8]

First, consideration of the mortality of mice subjected to different rates of production of decreased atmospheric pressure reveals that no effects definitely attributable to aeroembolism occur even with the most rapid rates of ascent. There is no sudden, large increase in lethal effects of exposure to low pressure other than the moderate influences related to failure of compensatory mechanisms antagonizing anoxia. However, to minimize further any possibility of aeroembolism, the proposed test utilizes a partial denitrogenation by exposure for ten minutes to a reduced pressure easily tolerated by the mice; i.e., a plateau at 10,000 feet. Absence of persistent aeroemboli in vessels of mice after drastic anoxic treatment, following return to normal atmospheric pressure, does not prove that aeroemboli do not occur at the height of anoxia, but all considerations indicate that aeroembolism is not an important factor even in mice subjected to rapid ascent.

Second, exposure to pure oxygen for ninety minutes before the mice are subjected to anoxia results in a significant denitrogenation of the tissues, probably of more than 95 per cent. Such treatment does not significantly reduce the lethal effects of anoxia as produced in the proposed test. Although there is no recognized reservoir for oxygen save the small effect of complete saturation of myohemoglobin,[12] minor physiologic effects of pretreatment with oxygen might be sufficient to explain the slightly increased tolerance of pretreated mice. Certainly there is not the major difference in mortality between pretreated and control mice which could be expected if aeroembolism were a major factor in the lethal effects of the conditions to which the mice are subjected in the proposed test.

Finally, pretreatment with helium-oxygen also does not significantly reduce lethal effects in mice. At atmospheric pressure helium is liberated from tissues about twice as fast as nitrogen and, therefore, should have less tendency to become supersaturated in blood and tissues during exposure to low pressures.[13] If aeroembolism were an important cause of death in the majority of the control mice exposed to the simulated high altitudes, pretreatment with helium-oxygen should significantly decrease this mortality.

It is to be noted that the possibility of an infrequent instance of aeroembolism occurring under the conditions of the proposed test is not ruled out conclusively by any one of the three factors discussed. Taken together, however, there is a strong indication that aeroembolism occurs so rarely, if at all, under these conditions that it does not invalidate interpretation of results obtained through a decompression technique as a true expression of tolerance of the mice to anoxia.

SUMMARY

A standard test of effects of drugs upon tolerance to anoxia is proposed. The method involves exposure of large groups of mice to low atmospheric pressures produced by a rigorously fixed technique in a decompression chamber allowing adequate ventilation, with simultaneous exposure of an equal number of untreated mice to the same conditions, and a comparison of mortality in control and treated groups by statistical criteria at the time 50 per cent of the control mice have died from anoxia. The function of this simple test is one of preliminary orientation, to obtain a measure of relative efficacies of different agents in com-

bating lethal effects of anoxia. For reasons discussed results cannot be considered applicable to therapy in man without further investigation. The influence of certain variable factors, including those which might affect the possible occurrence of aeroembolism, is studied. The results indicate that the test measures tolerance to anoxia and is not significantly affected by other factors.

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DRUG PROPHYLAXIS AGAINST LETHAL EFFECTS OF SEVERE ANOXIA

V. Agents Affecting the Autonomic Nervous System

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EFFECTS of anoxic anoxia on the activity of the autonomic nervous system have been summarized recently by one of us.[1] The bulk of the literature indicates that moderate or severe anoxia increases activity of the thoracolumbar division and evokes a discharge of epinephrine from the adrenal glands. The influence of anoxia on the craniosacral division is not as clear, although a compensatory stimulation might be expected even in the absence of direct effects. Monge[2] described an increased activity of the oculocardiac reflex in anoxic subjects, and others whom he cites have claimed that effects of atropine are diminished at high altitudes. Ury and Gellhorn[3] noted that in rabbits the threshold for reflex mydriasis by stimulation of the sciatic nerve is increased in anoxia, which may be interpreted as an indication of increased inhibition through the oculomotor nerve. Gasser and Loevenhart[4] found the cardioinhibitory center less susceptible to anoxia than the vasomotor center, both in the primary stimulation and subsequent depression. On the other hand, there is a controversy[1] as to whether tachycardia in anoxia is due to stimulation of the accelerator mechanism or to vagal depression. The recognized disturbance of sleep at high altitudes may perhaps indicate in part a preponderance of adrenergic over cholinergic stimulation by anoxia.

Since the work of Gomez and Pike,[5] several reports have emphasized the relative resistance of autonomic ganglia to structural damage by anoxia. Autonomic neurons are much less susceptible than the central nervous system. Thoracolumbar ganglia are more susceptible than Auerbach's plexus to loss of function in anemic anoxia. Medullary centers controlling automonic activity are more susceptible than the ganglia, and presumably the higher "centers" are still more susceptible.

Cats deprived of the larger part of their autonomic nervous system quickly die from degrees of anoxia ordinarily sublethal.[6] Thyroidectomy, which is said[7] to reduce susceptibility to anoxia, decreases the effects of epinephrine and increases cholinergic responses[8] while hyperthyroidism has the opposite effects.

Binet and Strumza[9, 10] and a few other observers have made direct studies of effects of several sympathicomimetic amines at lethal levels of anoxia. These reports are considered in the discussion of the present results.

A comprehensive survey of prophylactic actions of representative autonomic agents has been lacking and was, therefore, undertaken as a part of a series of

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studies designed to yield a preliminary evaluation of effects of different classes of drugs in anoxia for direction of more intensive pharmacologic study without which no clinical application of the results should be made. Maximal effects were desired in the present work, and conclusions as to the effects of clinical doses in man are not necessarily justified.

METHOD

A standardized technique of quantifying actions of drugs on lethal effects of anoxic anoxia was used. This method has been studied critically.[11] In brief, it depends on the comparison of mortality in groups of treated and control mice simultaneously exposed to identical conditions of anoxia produced in a manner which, we believe, avoids all such factors as aeroembolism which would invalidate the results.

Forty-one agents acting on the autonomic system or closely related to agents which do so were tested with small groups of mice. In 14 instances results were sufficiently suggestive to justify study with larger groups. The agents are divided into six classes, five of which are noted in Tables I to V. The sixth group is that of adrenergic sensitizers, of which cocaine was taken as the sole example.

TABLE I

EFFECT OF SYMPATHETIC STIMULANTS AND RELATED AGENTS ON RESISTANCE TO ANOXIA

AGENT	DOSE (MG./KG.)	MORTALITY RATIOS	
		TREATED MICE	CONTROL MICE
Phenethylamine hydrochloride	50	10/20	9/20
Phenethanolamine sulfate	50	8/10	5/10
l-Ephedrine hydrochloride	25	9/10	4/10
d-Pseudoephedrine hydrochloride	25	7/10	4/10
Hordenine sulfate	50	6/20	9/20
Tyramine (free base)	25	2/10	5/10
Propadrine hydrochloride	25	10/10	5/10
dl-Norephedrine hydrochloride	25	9/10	5/10
dl-Pseudonorephedrine hydrochloride	25	9/10	5/10
dl-Phenisopropylamine sulfate	10	50/50	11/50
	2	12/30	5/30
	0.2	18/30	9/30
l-Phenisopropylamine sulfate	10	47/50	11/50
	2	6/30	5/30
	0.2	15/30	9/30
d-Phenisopropylamine sulfate	10	50/50	11/50
	2	16/30	5/30
	0.2	11/30	9/30
Epinephrine hydrochloride	0.3	26/30	28/30
Mescaline hydrochloride	50	5/10	4/10
l-dihydroxyphenylalanine phosphate	100	7/10	5/10
Methylaminomethylheptene hydrochloride	50	9/10	5/10

TABLE II

EFFECTS OF SYMPATHICOLYTIC AGENTS ON RESISTANCE TO ANOXIA

AGENT	DOSE (MG./KG.)	MORTALITY RATIOS	
		TREATED MICE	CONTROL MICE
Ergotamine tartrate	5	6/25	15/25
Yohimbine hydrochloride	5	3/10	6/10
	1	5/10	6/10
Diethylaminomethylbenzodioxane hydrochloride	10	5/25	15/25
Piperidinomethylbenzodioxane	10	21/60	24/60
	1	6/10	4/10
Aspidospermine (cryst., Fraude)	5	6/10	6/10

TABLE III

EFFECT OF PARASYMPATHETIC STIMULANTS AND RELATED AGENTS ON RESISTANCE TO ANOXIA

AGENT	DOSE (MG./KG.)	MORTALITY RATIOS	
		TREATED MICE	CONTROL MICE
Acetylcholine iodide	20	5/10	4/10
Carbaminoylcholine chloride	5	3/10	5/10
Acetyl beta-methylcholine chloride	10	5/10	4/10
Neurine bromide	10	5/10	4/10
Physostigmine salicylate	1	2/18	9/20
	0.5	4/20	7/20
Prostigmine methylsulfate	0.5	15/15	1/20
	0.1	4/20	7/20
Pilocarpine hydrochloride	5	3/10	4/10
Arecoline hydrobromide	1	4/20	8/20
Betaine chloride	1,000	7/10	5/10

TABLE IV

EFFECT OF PARASYMPATHICOLYTICS AND RELATED AGENTS ON RESISTANCE TO ANOXIA

AGENT	DOSE (MG./KG.)	MORTALITY RATIOS	
		TREATED MICE	CONTROL MICE
Atropine sulfate	4	11/30	5/30
	2	6/10	5/10
Hyoscyamine sulfate	2	10/30	5/30
Duboisine sulfate	2	6/10	5/10
Hyoscine hydrobromide	0.5	5/10	5/10
Homatropine hydrochloride	5	7/10	6/10
Eucatropine hydrochloride	10	8/10	6/10
Trasentin	15	5/10	6/10

TABLE V

EFFECTS OF AGENTS ACTING ON AUTONOMIC GANGLIA ON RESISTANCE TO ANOXIA

AGENT	DOSE (MG./KG.)	MORTALITY RATIOS	
		TREATED MICE	CONTROL MICE
Nicotine (free base)	0.5	4/10	5/10
Lobeline sulfate	2	4/10	5/10
Sparteine sulfate	25	6/19	7/20

All agents were administered intraperitoneally. Those with prolonged effect were given thirty to sixty minutes before exposure of the mice to anoxia, while agents with brief duration of action were given within ten minutes of exposure. Lethal degrees of anoxia are not attained until some forty minutes after beginning exposure with the method used, however, so that results with short-acting agents must be interpreted cautiously.

RESULTS

Tables I to V present detailed results of treatment with different classes of agents affecting the autonomic nervous system, and Table VI is a summary of these data with the addition of results with cocaine which have been reported previously.[12] The mortality ratios signify the number of mice dying from anoxia divided by the total number of mice exposed. Mortality ratios for untreated control groups simultaneously exposed to anoxia are noted to the right of the corresponding treated groups. These bear no relation to mortality ratios of other control groups, since anoxia was terminated at different levels for different pairs

TABLE VI

SUMMARY OF EFFECTS OF GROUPS OF AGENTS ON RESISTANCE TO ANOXIA

GROUP	MORTALITY RATIOS		PER CENT MORTALITY	
	TREATED	CONTROLS	TREATED	CONTROLS
Sympathicomimetic agents (Table I)	342/500	168/500	68.4	33.6
Sympathetic sensitizer (cocaine)	13/20	11/20	65.0	55.0
Sympathicolytic agents (Table II)	52/150	76/150	34.7	50.7
Ergotamine, yohimbine, and 883F (Table II)	19/70	42/70	27.1	60.0
Parasympathicomimetic and related agents	57/153	58/160	37.3	36.3
Choline esters and neurine (Table III)	18/40	17/40	45.0	42.5
Other parasympathetic stimulants* (Table III)	17/88	35/90	19.3	38.9
Parasympathicolytics and trasentin (Table IV)	58/120	43/120	48.3	35.8
Ganglionic agents (Table V)	14/39	17/40	35.9	42.5

*Physostigmine, pilocarpine, arecoline, and prostigmine in a dose of 0.1 mg. per kilogram.

of treated and control groups and the resistance of mice to anoxia is not constant on different days.[12]

Following exposure to anoxia, the survivors were observed for twenty-four hours. No late effects of anoxia in treated mice were recognized.

Statistical treatment[13] of the combined data in Table VI demonstrates a significantly harmful effect of adrenergic and parasympathicolytic agents, and significant prophylactic effects of sympathicolytic agents and those with prolonged cholinergic effects. Short-acting cholinergic agents, those acting on autonomic ganglia, and the adrenergic sensitizer, cocaine, had no significant effects. The propriety of treating the combined data statistically may be questioned, but it is supported by the findings for individual agents. Significantly harmful effects were found for ephedrine, benzedrine, and its optical isomers, and propadrine, while significant prophylaxis was found with physostigmine, ergotamine, and the synthetic sympathicolytic, 883F. Other agents with similar actions would probably show significant effects if larger groups of mice were used.

DISCUSSION

A brief consideration of the properties of the individual agents is desirable, since none has a single, pure action on the autonomic nervous system. In several instances, lack of conformity with the general trend of the results is due to the short action of the agents in question. Otherwise, conclusions reached through consideration of results with groups of agents are generally supported for individual agents; that is, those with stronger adrenergic or parasympathicolytic activity tend to decrease resistance to anoxia more strongly, while cholinergic and sympathicolytic properties tend to prevent lethal effects of anoxia.

Adrenergic and related substances. Results with epinephrine and the isomers of benzedrine have been taken from previous reports[12, 14] in which their pertinent properties are adequately discussed. The sympathicomimetic action of epinephrine is the greatest of the agents studied, but this action is brief. Further, discharge of epinephrine from the adrenals at lethal levels of anoxia would make it unlikely that additional epinephrine or other sympathicomimetic agents would have desirable effects. Despite this improbability, ephedrine,

norephedrine, pseudonorephedrine, and benzedrine are claimed[9, 10] to delay respiratory failure in dogs subjected to fulminating anoxia. None has prophylactic value under the present experimental conditions. Synephrine, which is strongly adrenergic, is considered unsuitable[15] for the therapy of mountain sickness. These observations emphasize that agents which may be of temporary benefit in treating fulminating anoxia do not necessarily have any prophylactic value and may actually be harmful if used for prophylaxis. A study[12] of prophylactic effects of 12 respiratory stimulants confirms this. Conversely, agents such as alcohol with appreciable prophylactic activity are not necessarily suited for treatment of anoxia.[16]

Phenethylamine exerts mainly nicotine-like actions while phenethanolamine has a more purely adrenergic action.[17] Pseudoephedrine is weaker than ephedrine in most of its sympathicomimetic effects and is said to be a myocardial stimulant while ephedrine is depressant in full dose.[8] Hordenine causes vagal stimulation in small dose and paralyzes the vagus center in large dose but has definite adrenergic activity.[8] Tyramine may be considered as an adrenergic sensitizer similar to ephedrine; its effect in anoxia is not statistically significant. Norephedrine and pseudonorephedrine together constitute propadrine, of which the commercial preparation contains also a small amount of ammonium chloride. Despite the empirical use of ammonium chloride as a prophylactic against mountain sickness, propadrine is not less harmful than either of its fractions. Mescaline shares with benzedrine an enhanced activity on the central nervous system, but its effect in anoxia is not significant, although the stronger central stimulant isomer of benzedrine is significantly more harmful than the weaker isomer at one dose level.

Dihydroxyphenylalanine, "dopa," has no appreciable adrenergic properties but is related to the phenolic sympathicomimetic amines. Under the conditions of anoxia used, the redox functions of these agents are not of sufficiently long duration to act at lethal levels of anoxia. Octin, an aliphatic amine, has greater spasmolytic and less pressor effect than the true adrenergic agents, and its action in anoxia indicates that bronchodilatation is not an important factor in prophylaxis.

Sympathicolytic agents. The first three agents in Table II paralyze adrenergic functions for a relatively long period. The two dioxane derivatives act as mild narcotics in doses of 10 mg. per kilogram. Aspidospermine is a weak sympathicolytic and causes pulmonary edema and hyperemia.[8]

Cholinergic and related substances. The choline derivatives and neurine have a transient action. The remainder of the agents in Table III, except betaine, cause prolonged cholinergic effects. Physostigmine is most effective in anoxia in a dose within the lethal range, but prostigmine appears definitely harmful in high dose. Betaine is wholly inert as to cholinergic activity, but its chloride is strongly acid and is included as a control for the other salts used.

Belladonna group and trasentin. Although none of these agents has a significant harmful effect in anoxia, there is a close relationship between the parasympathicolytic potency and the detrimental effects in anoxia. Stimulant effects of these agents on respiration possibly mask the full effects of peripheral pa-

ralysis of the craniosacral nerves in anoxia. Hyoscine apparently does not act as a strong respiratory depressant in anoxia, and it is of interest that an antagonism of the respiratory effects of hyoscine and morphine has been noted clinically.[18] Properties of the five commonly used belladonna alkaloids need no further exposition. Duboisine is variously described as equivalent to hyoscyamine,[19] or to a mixture of hyoscyamine, hyoscine, and minor alkaloids.[20] Trasentin, or diphenylacetyldiethylaminoethanol hydrochloride, is not parasympathicolytic but is included as a control for the spasmolytic effects of the group. It may be noted that its effects in anoxia differ appreciably from those of octin.

Ganglionic agents. In addition to effects on autonomic ganglia, these agents have marked central effects, mediated in part through the carotid sinus mechanism. The resultant respiratory stimulation is brief. Thus, lobeline may be of some value in treatment of asphyxia neonatorum but is not prophylactic in anoxia. Sparteine affects autonomic ganglia but also potentiates epinephrine.[21] It acts as a myocardial poison and is included since it has been used clinically in conditions in which anoxia may occur; i.e., asthma and cardiac disease.

SUMMARY

Certain cholinergic and sympathicolytic agents have significant prophylactic actions against lethal effects of anoxic anoxia in mice, while adrenergic and parasympathicolytic agents tend to increase the lethal effects. Other classes of autonomic drugs are without appreciable effect. Differences in the therapeutic and prophylactic efficacies of individual agents are discussed.

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COMPARATIVE TOXICITY OF PENTOBARBITAL IN THE NEWBORN AND ADULT RAT*

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RECENT studies have disclosed that newborn rats, dogs, cats, and rabbits exhibit an extraordinary tolerance to anoxia[1] produced by the inhalation of pure nitrogen, helium, or carbon dioxide, or by the volatile narcotics, nitrous oxide and cyclopropane. The adult rat usually dies within a period of three minutes when respiring either pure nitrogen or these depressant drugs, whereas the newborn of the same species survives for approximately fifty minutes in nitrogen and nitrous oxide, and twenty-five minutes in cyclopropane and carbon dioxide. The newborn rats are also relatively resistant to alcohol[2] and to the acute lethal effects of morphine.[3] On the other hand, the newborn appears relatively more sensitive than the adult to the nonvolatile depressant drug, pentobarbital. Rosenfeld and Snyder,[4] and Windle and Becker[5] have shown that the barbiturates depress the respiratory efforts of the fetus more than those of the pregnant mother. In view of the discrepancy between the tolerance of the newborn to anoxia and the volatile anesthetics and the susceptibility of the fetus to barbiturates, the present comparative studies of the susceptibility and resistance of the newborn and adult rat to pentobarbital sodium were made. These observations include fetuses and pregnant rats near term.

In an effort to determine the mechanism of the action of barbiturates, the effect of pentobarbital on the survival time of the newborn in nitrogen and nitrous oxide was examined.

METHOD

Rats 1 to 2 days old and mature animals were injected intraperitoneally with various concentrations of pentobarbital. The tolerance of the newborn was

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compared with that of the adult animal given an equivalent concentration of barbiturate. In this manner the lethal dose for the newborn and the adult was determined. Cessation of respiration and inability to evoke respiration were taken as the criteria of the lethal effect.

Pregnant rats were subjected to various concentrations of pentobarbital. After allowing sufficient time for the absorption of the drug, cesarean section was done in order to study the effect on the fetus. One rat already in labor was injected with an anesthetic dose of pentobarbital, and the fetuses were observed after expulsion from the uterus. Infant rats subjected either to anesthetic or lethal doses of pentobarbital were placed in an atmosphere of pure nitrogen. The survival time of these newborn rats was compared with that of the uninjected litter mate controls. Other infant rats who had received lethal doses of pentobarbital were injected with various concentrations of metrazol to determine the combined effects of both drugs on respiratory depression and survival period. A control study was made to ascertain the tolerance of the newborn for metrazol.

TABLE I

SURVIVAL PERIOD OF NEWBORN AND ADULT RATS INJECTED WITH PENTOBARBITAL (0.1%)

NO. OF ANIMALS	AGE GROUP	PENTOBARBITAL (MG./GM.)	RESULTS	AVERAGE SURVIVAL TIME
3	Newborn	0.8	3 died	30 minutes
4	Newborn	0.5	4 died	35 minutes
4	Newborn	0.4	4 died	45 minutes
20	Newborn	0.3	16 died	60 minutes
8	Newborn	0.2	8 lived	Anesthetic dose
1	Adult	0.2	1 lived	No anesthesia
3	Adult	0.3	3 lived	No anesthesia
3	Adult	0.4	3 lived	Surgical anesthesia
6	Adult	0.5	6 lived	Surgical anesthesia
1	Adult	0.6	1 died	32 minutes
1	Adult	0.7	1 died	30 minutes

RESULTS

Table I reveals that 0.3 mg. per gram of body weight of pentobarbital is the minimal fatal dose for the rat 1 to 2 days old. Sixteen of 20 newborn animals died after an average survival period of sixty minutes, with a range of thirty to ninety minutes. All of 8 newborn animals receiving 0.2 mg. per gram survived, whereas 4 animals receiving 0.4 mg. per gram all died within forty-five minutes. The minimal fatal dose for adult rats of our colony is 0.5 mg. per gram of body weight. Each of 6 adult rats receiving this dose died after an average survival of thirty minutes. Adult rats receiving 0.3 mg. per gram were not even anesthetized. Those receiving 0.4 mg. per gram exhibited a short period of surgical anesthesia. In Table II is disclosed the sensitivity of fetal rats to pentobarbital. Three pregnant rats received pentobarbital in doses ranging from 0.3 mg. per gram to 0.4 and 0.5 mg. per gram of body weight. Twenty-nine fetuses were born spontaneously or were delivered by cesarean section fourteen minutes after the injection of the drug. All fetuses died from ten to forty minutes after the delivery, and the extrauterine survival time decreased with increasing dosage of pentobarbital. In contrast all the mothers with the exception of one which received 0.5 mg. per gram survived.

TABLE II

EFFECTS OF PENTOBARBITAL (0.1%) ON FETAL RATS

NO. OF PREGNANT RATS	PENTOBARBITAL (MG./GM.)	PROCEDURE	NO. OF FETUSES	RESULTS	SURVIVAL PERIOD OF FETUSES
1	0.3	Cesarean section	9	9 died	40 minutes
1	0.4	Cesarean section	10	10 died	10 minutes
1	0.5	Section after death	10	10 died	10 minutes
1	0.3	Spontaneous delivery	4	3 died	45 minutes

TABLE III

EFFECTS OF PENTOBARBITAL (0.1% AND 0.3%) AND ANOXIA ON SURVIVAL PERIOD OF NEWBORN RATS

NO. OF NEWBORN RATS	PENTOBARBITAL (MG./GM.)	AGENT	RESULTS	AVERAGE SURVIVAL TIME
3	0.1	Nitrogen	3 died	50 minutes
3	0.1	Nitrous oxide	3 died	50 minutes
2	0.1	Nitrogen	2 lived	40 minutes
6	0.3	Nitrogen	6 died	25 minutes

A rat which had delivered 4 of her fetuses was injected with 0.3 mg. per gram of pentobarbital. Eighteen minutes after the injection one fetus was born spontaneously. It exhibited no signs of respiratory depression and survived. Three fetuses born from forty-five to ninety minutes after the injection were in apnea. Respirations could not be evoked and all 3 died. Table III presents the data on infant rats placed in an atmosphere of nitrogen or nitrous oxide after receiving 0.1 mg. per gram, an anesthetic dose, or 0.3 mg. per gram, a lethal dose, of pentobarbital. Six newborn rats receiving the lower dose survived for fifty minutes either in nitrogen or nitrous oxide. However, the infant rats with the larger dose survived on an average only twenty-five minutes.

TABLE IV

EFFECTS OF METRAZOL (10 PER CENT) ON SURVIVAL PERIOD OF NEWBORN RATS

NO. OF NEWBORN RATS	METRAZOL (MG./GM.)	EFFECT	RESULT
6	5.0	Convulsion	6 died
5	4.0	Convulsion	5 died
5	3.0	Slight convulsion	5 lived
5	2.0	Slight convulsion	5 lived

Table IV shows the results of the experiments made to determine the lethal dose of metrazol for newborn rats. Five animals injected with 3.0 mg. per gram of body weight exhibited mild convulsive movements and survived. With larger doses 4.0 and 5.0 mg. per gram the animals died as a result of the severe convulsions.

TABLE V

EFFECT OF PENTOBARBITAL (0.3 MG./GM. BODY WEIGHT) AND METRAZOL (10%) ON SURVIVAL PERIOD OF NEWBORN RATS

NO. OF NEWBORN RATS	METRAZOL (MG./GM.)	INJECTION TIME AFTER PENTOBARBITAL	RESULT
5	7.5	20 minutes	5 lived
5	5.0	15 minutes	5 lived
5	4.0	22 minutes	5 lived
5	3.0	35 minutes	5 lived

Table V presents the effects of various concentrations of metrazol on infant rats given a lethal dose of pentobarbital. It may be seen that 3.0, 4.0, and 5.0 mg. per gram of metrazol successfully antagonize lethal doses of pentobarbital.

DISCUSSION

In contrast with the extraordinary tolerance of the newborn rat to the volatile anesthetics, nitrous oxide and cyclopropane, the infant rat both ante partum and post partum is more sensitive to pentobarbital than is its mother. The newborn rat died with a dose of 0.3 mg. per gram and the adult with 0.5 mg. per gram. Pentobarbital depresses the respiratory centers so that the animal cannot be resuscitated and dies from anoxia. Since the newborn rat is more tolerant of anoxia than is the adult, the greater sensitivity of the infant rat to barbiturate cannot be explained simply by the anoxia produced as a result of the depression of respiration. The mechanism which normally facilitates the survival of the newborn rat during anoxia depends upon the anaerobic splitting of carbohydrate with the formation of lactic acid.[6, 7] It is true that nonfatal doses of barbiturate do not shorten the survival period of the newborn rats in an atmosphere of nitrogen. However, a fatal dose of pentobarbital (0.3 mg. per gram) which permits the infant rat to survive for sixty minutes in air cuts down the survival period in nitrogen to twenty-five minutes. This is half the period in which the infant rat can survive in pure nitrogen. It would, therefore, seem that pentobarbital inhibits the mechanism for the anaerobic production of energy from carbohydrate. One path of the breakdown of carbohydrate involves a series of the same intermediates from glucose to the formation of pyruvic acid irrespective of the presence or absence of oxygen. Pyruvic acid, however, is then either oxidized to carbon dioxide and water, or in the absence of oxygen is converted to lactic acid. It would, therefore, seem that pentobarbital interferes with some stage in glucose metabolism before the formation of pyruvic acid. In any case lactic acid formation is not increased by the barbiturates.[8] Quastel and Wheatley[9] have previously indicated that barbiturates depress aerobic sources of cerebral energy. The present evidence reveals that barbiturates probably also inhibit the anaerobic breakdown of carbohydrate.

Though the newborn rat is susceptible to pentobarbital, it is able to withstand a lethal dose with the aid of metrazol. The mutual antagonism between the two drugs is emphasized because the dose of metrazol used would have been lethal for an animal that had not received pentobarbital.

CONCLUSIONS

1. The newborn rat is more susceptible than the adult to pentobarbital both ante partum and post partum. The lethal dose for the newborn rat is 0.3 mg. per gram, and for the adult 0.5 mg. per gram.

2. Evidence is afforded indicating that pentobarbital inhibits the development of the anaerobic energy required for survival in the absence of oxygen.

3. Doses of pentobarbital and metrazol, which when given singly are lethal, may successfully antagonize each other and facilitate survival when administered together.

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SPECIES OF THE TRIBES MIMEAE, NEISSERIEAE, AND STREPTOCOCCEAE WHICH CONFUSE THE DIAGNOSIS OF GONORRHEA BY SMEARS*

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FORTY years ago gram-negative diplococci from spinal fluids[1] or conjunctivitis[2] were considered meningococci. Such statements were refuted[3] at the time and since then it has been proved quite conclusively by other investigators[4-6] that diplococci other than the meningococci can cause a meningitis. Also during that time gram-negative diplococci in smears from conjunctivitis and vaginitis have been considered gonococci, although gram-positive diplococci which lose the Gram stain,[7, 8] lactose-fermenting rods which appear microscopically to be gram-negative diplococci,[9] and a species of the genus Neisseria other than the gonococcus in a rape case[10] have been reported. The investigations of Rossiter[11] on Samoan conjunctivitis were probably the first which proved definitely that a gram-negative diplococcus indistinguishable from the gonococcus in smear preparations was present in one type of conjunctivitis. This conjunctivitis had been diagnosed previously by smear preparations as gonorrheal despite the fact that venereal gonorrhea was not known on the island at that time.

The present investigation was undertaken to study the flora of conjunctivitis and vaginitis with special reference to the gram-negative diplococci. The cases studied included the newborn; children with conjunctivitis; girls of prepuberty age, both normal and those with varying degrees of vaginitis; and one case of conjunctivitis in an adult male.

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EXPERIMENTAL

The medium used for isolation was a chocolate agar made from Difco proteose peptone No. 3 with 5 per cent citrated human blood. Later rabbit blood was substituted for human blood. Concentrated sugar solutions in distilled water were sterilized at 15 pounds pressure for fifteen minutes and added aseptically so that the final dilution in broth was 1 per cent. Acetone was used as the decolorant in the Gram stain. All media which were not used as they came from the autoclave were incubated for twenty-four hours before use. A sterile cotton swab was used to obtain the specimen which was streaked immediately over the plate. Each type of colony, with some duplications, was examined microscopically for gram-negative diplococci. The examinations for Neisseriae were made by the oxidase test with dimethyl-p-phenylenediamine. Many species, especially gram-negative rods, give this test, which makes it essential to examine the colonies microscopically and culturally for the correct determination of the species if gram-negative diplococci be present. This confirms the work of Gordon and McLeod[12] and Price.[13]

One hundred and forty-seven cases were studied. These included 41 patients with purulent conjunctivitis but did not include many other cases in which the eyes showed a mild discharge with a few intracellular or extracellular gram-negative diplococci in the direct smears and no species of Neisseria on the plates. Of the 41, 10 were infections with *Neisseria gonorrhoeae,* two cases showed *Neisseria catarrhalis,* and one showed *Neisseria fulva.*[14] The one adult male case showed *Neisseria intracellularis.* This species was shown to be serotype I when typed by Dr. Sara Branham of the U. S. Public Health Service. Twelve cases showed species of the tribe Mimeae,[14] of which two were *Herellea vaginicola* and *Colloides anoxydana.* Species of the remaining cases were not classified except to show that Neisseriae were not present.

Forty-nine cases showed vaginitis. Five were infections with *N. gonorrhoeae. N. fulva* was isolated once, three cases showed *N. catarrhalis,* and nine cases showed species of the tribe Mimeae, two of which were *Mima polymorpha* var. *oxidans* and one was *C. anoxydana.* The species of the remaining cases were not investigated further than to show that no species of the genus Neisseria was present.

In the group of 57 clinically normal cases, many showed an occasional intracellular or extracellular gram-negative diplococcus in the smear. One case showed *Neisseria gigantea,*[14] two showed *N. catarrhalis,* and 24 cases showed Mimeae of which two were *Mima polymorpha,* two were *M. polymorpha* var. *oxidans,* and one each of the species *H. vaginicola* and *C. anoxydana.*

DISCUSSION

The presence of gram-negative diplococci which were not Neisseriae was found by a systematic search. This was done by making a stained smear of every type, with some duplications, of colonies on the plate. Those species which appeared to be gram-negative diplococci were then studied culturally. The visual examination of the colonies is not sufficient because it is not possible to differentiate Mimeae, many forms of the gram-positive diplococci, and many forms of the diphtheroids by their colonial characters on plates. The three tribes,

Neisserieae, Streptococceae, and Mimeae, represented by the genera Neisseria, Mima, Herellea, Colloides, and the gram-positive diplococci which tend to lose the stain were found to be present in conjunctivitis and vaginitis. All these species are either true gram-negative diplococci or have forms which are gram-negative diplococci.

The presence of species of the genus Neisseria other than *N. gonorrhoeae* must be considered when diagnoses are made upon the basis of morphology. While these species are relatively less important when judged by the number which have been found in conjunctivitis and vaginitis, the possibility of their presence is one factor which must be considered when evaluating the reliability of smear examinations. The presence of *N. intracellularis* in the one case could have been detected only by cultural methods, thereby enhancing those methods in conjunctivitis. *N. intracellularis* and *N. fulva* were present in practically pure culture in both cases in which they occurred. The presence of *N. gigantea* could explain the statements in the literature that some cultures of the gonococcus showed large forms. The inability of *N. gigantea* to ferment sugars would not permit its presence to be recognized as a contaminant of cultures of *N. gonorrhoeae* because the fermentative reactions of *N. gonorrhoeae* would not be changed. Only the microscope would reveal the larger form. *N. catarrhalis* and *N. gigantea* were present in relatively few numbers in the cases in which they were found.

A second group of minor importance is the gram-positive diplococci which tend to lose the Gram stain. Schubert and Toenges[7] included certain streptococci in this group also. These species may be present in either vaginitis or conjunctivitis. The number of cases involved is low, and a systematic search by staining smears from many individual colonies on the plate is necessary to find those which decolorize, although they do predominate occasionally. The gram-positive diplococci which tend to lose the stain cannot be differentiated on the plates from those which do not decolorize because their colonial characters are the same and those which do not decolorize may be present in large numbers in vaginitis and conjunctivitis. The pairs of cocci which have decolorized are identical in appearance to the gonococcus when the size is the same.

The most important group is the tribe Mimeae. The prevalence of this tribe complicates the microscopic picture to such an extent that it is imperative to use cultural methods to obtain a true evaluation of the flora.

Since the microscopic picture of the species of the tribe Mimeae is identical to the genus Neisseria, except *N. gigantea,* all species of the tribe interfere in the diagnosis of gonorrhea by smears and *M. polymorpha* var. *oxidans* can interfere in the cultural method when tests are made presumptively by the oxidase test for the presence of Neisseriae.

The question of pathogenicity with these species cannot be answered categorically because Koch's postulates have not been fulfilled with the original animal, the human being. There are, however, several points indicating that they may be primary or secondary invaders. First, the diplococcal form is the more prominent at the primary isolation and the diplococcus/rod ratio[14] falls upon artificial cultivation. Second, extensive animal inoculations have not been done, but species having low diplococcus/rod ratios injected intraperitoneally in rela-

tively large numbers have been recovered with high ratios from the heart blood of the mice after death. Third, in conjunctivitis in the newborn where the data can be obtained, the infection arises very consistently from the fifth to the seventh day after birth and has persisted for as long as two months. Fourth, a change from an unbuffered silver nitrate solution to a buffered one used in the eyes at birth reduced the number of cases materially. Fifth, with all other supporting evidence lacking, pathogenicity might be assumed when only one species is present in the infection. This condition has not always been true, but in the cases with *N. intracellularis, N. fulva,* and some of the Mimeae in conjunctivitis, also with *N. fulva* and one case with *M. polymorpha* var. *oxidans* in vaginitis, the number of colonies of other species on the plate was negligible. It was noted that the species of Mimeae with greater fermentative powers, some of which have not been described, were more prevalent in conjunctivitis and vaginitis than those of the genus Mima which does not ferment carbohydrates. Proof is lacking whether this might be due to a greater pathogenicity or merely accidental circumstances. Whether any of these species are pathogenic or not, they constitute important sources of error which confuse the diagnosis of gonorrhea by smears.

As a result of the probable presence of one or more of the three groups, Mimeae, Neisserieae, gram-positive diplococci which tend to lose the stain, it is impossible to make logical comparisons between direct smears and cultures in conjunctivitis and vaginitis. In the smears the gram-negative diplococci of the three groups are not differentiated from the gonococcus, rods and filaments cannot be used as a basis for determining the presence of the pleomorphic Mimeae, and gram-positive diplococci would not prove whether it was a species that tended to lose or retain the stain. There is, therefore, no criterion which would indicate the presence of these species in stained preparations, and it would be entirely possible for a diagnosis of gonorrhea to be given from a smear preparation in conjunctivitis or vaginitis, even though a pure culture of either Mimeae or gram-positive diplococci which tend to lose the stain was present. The Mimeae, with the exception of *M. polymorpha* var. *oxidans,* and the gram-positive diplococci which tend to lose the stain would not be detected by the oxidase test on the plate, and their presence could be determined only by a systematic search for gram-negative diplococci.

The statement that the gonococcus is difficult to grow may be explained by the presence of one or more of the tribes, Mimeae, Neisserieae, Streptococceae, which show as gram-negative diplococci in stained preparations but could not be detected readily on the cultural plate unless a systematic search were instituted for them. Neither the presence of gram-negative diplococci in a stained smear nor the oxidase reaction on a plate proves the presence of *N. gonorrhoeae.*

SUMMARY AND CONCLUSIONS

From the 147 cases of conjunctivitis, vaginitis, and normal vaginas, Mimeae were isolated forty-five times; gram-positive diplococci, which tend to lose the stain, six times; and Neisseriae, excluding the gonococcus, eight times. The total, 59, represents about 40 per cent in which real or apparent gram-negative diplococci were present which can be mistaken easily for *Neisseria gonorrhoeae.*

As a result of this investigation it is felt that the identification of *N. gonorrhoeae* by smears in conjunctivitis or vaginitis is not justified.

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EFFECT OF ETHYL ALCOHOL INTOXICATION ON THE DEVELOPMENT OF LOCAL INFLAMMATORY REACTIONS IN THE RABBIT

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PICKRELL[1] in 1938 reviewed the literature on the effect of intoxication on infection and immunity. He states that "while it is rather generally agreed that infected intoxicated animals die sooner than similarly infected, nonintoxicated ones, it is fair to say that very few of the studies have been adequately controlled. . . . No satisfactory explanation has as yet been established for any resistance lowering effect that alcoholic intoxication may exert."

From an experimental study made upon a large series of rabbits, Pickrell[1] concludes that "alcoholic intoxication maintained at the point of stupor, de-

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stroys resistance to pneumococcal infection. . . . The loss of resistance to infection appears to be due to the fact that intoxication profoundly inhibits the vascular inflammatory response. . . . In the absence of capillary dilatation and margination of the leucocytes, leucocytic emigration at the site of infection is negligible. . . . In what manner intoxication acts to prevent the usual inflammatory alterations in the capillaries is at present obscure but that it does so is clearly evident."

Cressman and Rigdon[2] studied capillary permeability and inflammation in narcotized rabbits and found that "capillary permeability in areas of inflammation is altered in rabbits narcotized with alcohol or ether, as demonstrated by the localization and concentration of trypan blue. . . . There is also a marked diminution in the number of leucocytes in the areas of inflammation in the skin in the narcotized rabbits." The observations of Cressman and Rigdon, therefore, are similar to those of Pickrell; however, neither have explained adequately the mechanism of this phenomenon.

Rigdon and associates have recently demonstrated that capillary permeability and inflammation in the skin of the rabbit may be affected by changes in the blood pressure; that is, when the pressure is abnormally low, the macroscopic and microscopic changes characteristic of inflammation either fail to appear or they are markedly decreased in the skin. It has been suggested that the peripheral vessels in the skin become constricted following the loss of blood,[3] sectioning of the cervical portion of the spinal cord,[4] and in staphylococcus septicemia in the rabbit.[5] The blood apparently does not circulate in the small blood vessels in the skin of these animals since the skin becomes pale, cold, and clammy. This absence of a normal circulation apparently may account for the absence of a leucocytic reaction about various irritants when they are injected intradermally into the animals.

Alcohol in large amounts will produce a lowering of the blood pressure.[6] This change in the pressure apparently may result from the effect of the alcohol on the heart. In view of our present knowledge of the effect of lowered blood pressure on the development of an inflammatory reaction in the skin of the rabbit, it seems important to restudy the effect of ethyl alcohol intoxication on the development of the inflammatory reaction in the skin of the rabbit. Observations are also made on the development of the inflammatory process in the rabbits' intestines since the circulation here apparently remains intact while that in the skin may become either markedly decreased or even absent in severe intoxication.

METHODS AND MATERIALS

Adult rabbits, varying from 1.5 to 2.5 kg. are used. The skin over the abdomen is carefully shaven twenty-four hours or longer before the experiments are begun. A 20 per cent dilution of ethyl alcohol is given through a small catheter into the stomach. Each rabbit is given 80 c.c. of alcohol, and smaller quantities are given subsequently if necessary to obtain complete anesthesia. The blood pressure is recorded at intervals from the carotid artery.

Four-tenths cubic centimeter of a twenty-four-hour broth culture of staphylococci are injected intradermally usually six, four, and two hours before the rabbits are killed. An incision is made in the abdominal wall; the intestines are exposed, and 0.4 c.c. of the broth culture is injected into the wall of the colon

of some of these rabbits. The animals are killed from two to six hours following the injection of the staphylococci. The areas of tissue injected with the cultures are removed and fixed immediately in a 10 per cent solution of formalin. Paraffin sections are prepared and they are stained with hematoxylin and eosin.

To study the effect of alcohol intoxication on capillary permeability, the abdomen of a group of rabbits is exposed to infrared radiation.* The light is placed 10 inches away from the skin. Immediately following the application of the light, 10 c.c. of a 0.2 per cent solution of trypan blue are given intravenously. This dye localizes and concentrates in the skin around the margin of the irradiated areas in normal rabbits and also in the coils of intestines that lie immediately beneath the portion of abdominal wall that is irradiated. The blood pressure is recorded from the carotids in these animals.

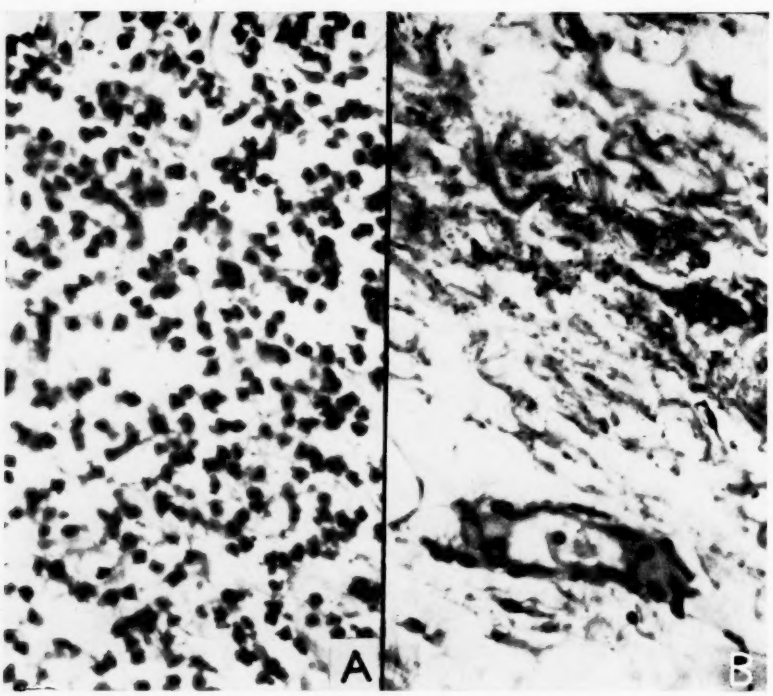

Fig. 1.—*A*, Rabbit 1192. Normal rabbit, 0.4 c.c. of twenty-four-hour broth culture injected intradermally six hours before section removed. There is a diffuse infiltration of polymorphonuclear leucocytes in the corium. *B*, Rabbit 1213. Rabbit given 70 c.c. of 20 per cent alcohol at 8:40 A.M.; blood pressure 80 at 9:50 A.M.; 0.4 c.c. broth culture of staphylococci injected intradermally at 10:20 A.M.; blood pressure 60 at 1:00 P.M., and 40 at 4:00 P.M. Rabbit is killed at 4:30 P.M. The corium is infiltrated with bacteria; but there are no leucocytes infiltrating the area.

As a control for this experiment on capillary permeability one group of rabbits is given pentobarbital intravenously; a second group is unanesthetized except for using 1.0 c.c. of a 1.0 per cent solution of novocaine in the neck before putting the cannula into the carotid artery. Infrared irradiation and trypan blue are given in a manner similar to that in the experimental group. The presence or the absence of trypan blue in the skin is recorded. The rabbits are killed at intervals varying from thirty minutes to four hours following the injection of the dye.

*The infrared lamp is supplied by the Burdick Company of Milton, Wisconsin. It is type Z-70, Zoalite.

LOCALIZATION OF POLYMORPHONUCLEAR LEUCOCYTES ABOUT STAPHYLOCOCCI INJECTED INTRADERMALLY IN NORMAL AND INTOXICATED RABBITS

Five normal rabbits are injected intradermally with the broth culture of staphylococci. Edema and hyperemia develop around the site of the injection. Polymorphonuclear leucocytes infiltrate the tissues within a period of two hours. The number of leucocytes rapidly increase during the subsequent four hours (Fig. 1*A*).

Sixteen rabbits are given alcohol. When completely anesthetized, a cannula is put into the carotid artery and the pressure is recorded. It is usually decreased at this time from the normal of 100-120 mm. Hg. The pressure may continue to fall or it may be necessary to give a second dose of alcohol in order to lower the pressure. The bacteria are not injected until the pressure reaches a level of approximately 70 mm. Hg or even less. The macroscopic reaction in the skin apparently is proportional to the level of the blood pressure. When the pressure is approximately 60 mm. Hg, the edema and hyperemia are markedly reduced. There is essentially no macroscopic reaction when the pressure is either 50 mm. or less. Histologically the number of polymorphonuclear leucocytes are proportional to the level of the blood pressure. When the pressure is either 50 mm. Hg or less, there are essentially no polymorphonuclear leucocytes about the clumps of bacteria (Fig. 1*B*).

LOCALIZATION OF POLYMORPHONUCLEAR LEUCOCYTES ABOUT STAPHYLOCOCCI INJECTED INTO THE WALL OF THE COLON OF RABBITS INTOXICATED WITH ETHYL ALCOHOL

In the previous experiments it was shown that polymorphonuclear leucocytes fail to localize and to concentrate about groups of staphylococci in the skin of rabbits intoxicated with ethyl alcohol when the blood pressure is abnormally low. A group of six rabbits are given a quantity of alcohol equal to that given to the animals in the preceding experiments. Staphylococci are injected into the wall of the colon and into the skin of these rabbits. The blood pressure is recorded during a period of four to six hours subsequently. When the pressure is abnormally low, there are no macroscopic evidences of inflammation in the skin. There are macroscopic evidences of inflammation, however, in the intestines about the point of injection of the bacteria. Histologically, there are no polymorphonuclear leucocytes about the bacteria in the skin, although many leucocytes have infiltrated the wall of the intestines about the point of injection of the bacteria (Fig. 2).

The results of this experiment indicate that intoxication per se is not the direct cause of the failure of leucocytes to localize and to concentrate about collections of staphylococci. If the bacteria are in the wall of the colon, the leucocytes localize and concentrate in a manner similar to that in an unintoxicated animal. Furthermore, the results suggest that the reaction in the skin is directly affected by the level of the blood pressure. When the pressure is abnormally low, the skin becomes pale, cold, and clammy, indicating a disturbed circulation in the peripheral vessels. The blood flow through the intestine of the same rabbits apparently is not similarly decreased. Polymorphonuclear leucocytes continue to localize and to concentrate about staphylococci as they do in an unintoxicated rabbit.

OBSERVATIONS ON CAPILLARY PERMEABILITY IN THE SKIN AND INTESTINES OF INTOXICATED RABBITS

In unpublished experiments it has been found that infrared radiation when applied to the skin of a normal rabbit for five minutes produces both macroscopic and microscopic evidences of inflammation. Similar pathologic changes also occur in the wall of the intestines. Trypan blue injected intravenously into these rabbits localizes and concentrates in both the treated areas of skin and in the intestines.

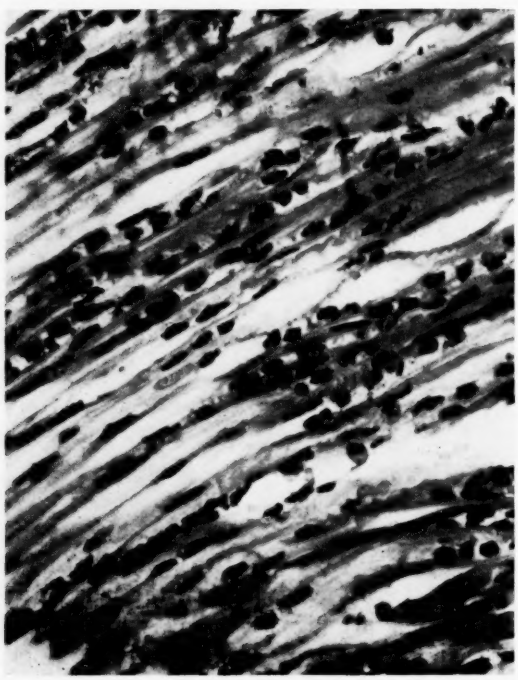

Fig. 2.—Rabbit 1213. Same rabbit as shown in Fig. 1 *B*. 0.4 c.c. of the broth culture of staphylococci was injected into the wall of the colon six hours before the rabbit was killed. There are many leucocytes infiltrating the area.

Fig. 3.—Rabbit 1294. Rabbit given 80 c.c. of alcohol at 9:30 A.M. Blood pressure 80 at 10:15, 60 at 10:30, 35 at 11:05, and 30 at 11:10. Infrared light is applied for five minutes to skin of abdomen in area outlined with India ink at 11:20; 10 c.c. of trypan blue are given intravenously immediately following the light. At 12:00 there is no trypan blue in the skin, but the portion of intestines immediately beneath the treated area of skin is dark blue in color.

In the study of the effect of alcohol intoxication on capillary permeability six rabbits are given alcohol. The blood pressure is recorded. When it is abnormally low (approximately 50 mm. Hg), the infrared lamp is placed over the abdomen for a period of five minutes. The macroscopic reaction in the skin is insignificant. The animals are given trypan blue intravenously immediately following the irradiation. A minute amount of the dye localizes in the skin of some of the rabbits; however, no dye localizes in the skin of the majority of the animals (Fig. 3). The coils of intestines beneath the site of application of the light becomes blue in every rabbit (Fig. 3).

Three rabbits are given pentobarbital in a quantity sufficient to produce complete anesthesia. The blood pressure is recorded from the carotid. It is 80, 80, and 85 in each of the three animals. Infrared is applied to the abdomen. Trypan blue is injected intravenously. This dye localizes around the periphery of the area of skin treated and in the underlying portion of the intestines. The normal blood pressure in a second group of three rabbits is determined before the infrared irradiation is applied. Local anesthesia is used (1.0 c.c. of 1 per cent novocaine) for the operative procedure necessary to put the cannula into the carotid. The pressure in these three rabbits is as follows: 90, 90, and 95. Trypan blue localizes in the skin and in the intestines of each of these rabbits. These two groups of rabbits are controls for the above experiment and show that this dye will localize in the skin provided the pressure has not reached the abnormally low level of 50 mm. Hg.

The results of this experiment show that trypan blue may fail to localize and to concentrate about areas of injury in the skin of rabbits intoxicated with ethyl alcohol; however, this dye always localizes and concentrates in the intestines of each of these intoxicated animals.

DISCUSSION

The results of this experiment confirm the observations of Pickrell,[1] and Cressman and Rigdon[2] that alcohol intoxication may completely inhibit the development of an inflammatory reaction in the skin of the rabbit. The data suggest that this phenomenon does not result directly from the effects of alcohol upon either the leucocytes or the local blood vessels. The circulation in the skin apparently is markedly reduced in intoxicated rabbits, and as a consequence of this trypan blue and polymorphonuclear fail to localize in the skin of these animals. The fall in blood pressure, the rapid rate of the respirations, the cold, clammy skin all indicate that a diminution has occurred in the blood flow through the peripheral vessels.

The circulation in the intestinal tract during intoxication is not affected similarly to that of the skin. Capillary permeability here is increased, and leucocytes localize about foci of bacteria the same as they do in a normal rabbit. Edmunds and Gunn[6] state that large quantities of alcohol lower the blood pressure by weakening the vasoconstrictor centers and the heart muscle. In a series of experiments it has been shown that a leucocytic reaction fails to occur in the skin of rabbits with a low blood pressure.[3-5] The results of the present experiment are consistent with these observations upon capillary permeability and the development of local inflammatory reactions.

SUMMARY

Polymorphonuclear leucocytes fail to localize and to concentrate about staphylococci in the skin of rabbits intoxicated with ethyl alcohol when the blood pressure is abnormally low.

Leucocytes concentrate about staphylococci injected into the colon of the same intoxicated animal. This variation in the reaction in the skin and the colon may result from the lowered pressure produced by alcoholic intoxication. When the pressure is lowered, the skin becomes cold and clammy, suggesting that the peripheral circulation is markedly decreased or completely inhibited. The circulation in the intestines apparently remains intact.

Capillary permeability as shown by the localization of trypan blue following an intravenous injection is absent in areas of injured skin in rabbits intoxicated with ethyl alcohol. It is present, however, in the injured areas of intestines of the same intoxicated rabbit.

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ALCOHOLIC CIRRHOSIS OF THE LIVER*

A Clinical and Pathologic Study of 356 Fatal Cases Selected From 12,267 Necropsies

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SINCE 1826 when Laënnec adequately described cirrhosis of the liver, it has been observed and recognized, without much difficulty, both clinically and pathologically. However, the observation of increased connective tissue stroma in livers of varying weights, sizes, shapes, colors, and histologic architectures has provoked a certain amount of confusion in the nomenclature. As a result, in common usage today are such terms as atrophic cirrhosis, hypertrophic cirrhosis, multilobular cirrhosis, portal cirrhosis, biliary cirrhosis, Laënnec's cirrhosis, Hanot's cirrhosis, hobnail liver, alcoholic cirrhosis, gin drinker's liver, toxic cirrhosis, Wilson's disease, Banti's disease as well as many others.

Broadly, cirrhosis of the liver is merely a condition in which the connective tissue stroma is increased. Any stimulus to fibroblastic proliferation may eventually produce a well-marked cirrhosis. Mallory[1] lists four conditions under which the fibroblasts of the liver stroma proliferate:

1. To replace any which have been destroyed as a result of abscess, infarcts, syphilis, colon bacillus infection, etc.
2. Following mechanical injury (stretching), as in chronic passive congestion and bile stasis.
3. To organize fibrin, as for thrombi in sinusoids.
4. To form stroma for islands of regenerated liver cells and for tumor nodules.

It is apparent that the number of agents which may initiate the ultimate picture of cirrhosis is very large and for that reason the question "Is cirrhosis of the liver a disease entity?" is often raised.

The great majority of cirrhotic livers fall into four major groups and can be classified as follows:

1. Toxic cirrhosis
2. Biliary cirrhosis
    a. Obstructive
    b. Infectious
3. Pigment cirrhosis
4. Alcoholic cirrhosis
    a. Periportal cirrhosis
        I. Atrophic form (small liver)
        II. Hypertrophic form (large liver)

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b. Fatty cirrhosis (hypertrophic form)
(Large amount of fat and little or no fibrosis)

Though this classification does not include every type of sclerotic liver known, many of which are exceedingly rare, it does encompass the commonly known types and does, more or less, conform to the nomenclatures and classifications of other investigators.[3-5]

Toxic Cirrhosis.—This occurs after necrotizing lesions of the liver parenchyma and may follow acute infections, such as acute yellow atrophy, repeated attacks of catarrhal jaundice, Weil's disease; or it may be the result of the ingestion of such drugs as cinchophen or arsenic.

Biliary Cirrhosis.—The obstructive type is associated with obstruction of the biliary passages by stone or tumor; the infectious type begins as an ascending cholangitis. These two types frequently co-exist.

Pigment Cirrhosis.—In hemachromatosis, often called "bronzed diabetes" because of the association of the typical coloring of the skin with glycosuria, the cirrhosis of the liver is merely one of the manifestations of a generalized condition. Either as a result of the liver-cell necrosis or as a result of the irritation of the iron pigment collected around the periphery of the lobule, there is an active macrophage and fibroblastic proliferation from the periportal connective tissue.

Alcoholic Cirrhosis.—The term "alcoholic cirrhosis" is an arbitrary one and, perhaps, not a very good one. To limit the diagnosis of alcoholic cirrhosis to those cases in which a definite history of chronic alcoholism is given, as Boles and Clark[6] do in their report of autopsy material from the Philadelphia General Hospital, we feel is too strict. The fatty cirrhosis is conceded by most investigators to be of alcoholic origin. The Laënnec cirrhosis is the type that has been called "alcoholic" for over a hundred years. There is a third type of alcoholic liver, the so-called "hypertrophic fatty form." It is large, tense, and loaded with fat. The liver is swollen and greasy; it may appear granular due to the subsurface lobules being distended with fat (see Fig. 1). This type of liver usually shows little or no sign of fibrosis and is observed in young people between 30 and 40 years of age. This form is usually larger than normal, smooth in contour, and weighs about 3,000 Gm. It may, however, be lobulated and nodular, depending upon the amount of connective tissue present. On cutting it is found to be greasy yet resistant.

Periportal cirrhosis is the typical portal cirrhosis which was described by Laënnec. The liver may be large or small, smooth or granular. The thought that it is larger in its early stage and becomes atrophic has been discarded. The noxious agent which initiates the cirrhosis is thought to enter the liver by way of the portal blood stream. The adjacent liver cells are damaged and connective tissue proliferation results. The newly proliferated connective tissue may become condensed and sclerotic, producing the small, hard, hobnail liver. Because of obstruction to the portal circulation, ascites develops, the spleen becomes engorged, the mucosa of the gastrointestinal tract becomes congested, and attempts at the establishment of a collateral circulation to compensate for the decreased venous bed are apparent. The presence of a caput medusae on the anterior abdominal wall and the frequent hemorrhages resulting from rupture of dilated

and varicose veins in the lower esophagus are typically products of this common type of cirrhosis.

The association of cirrhosis of the liver with alcohol is still a debatable point. Attempts at the production of cirrhosis experimentally with alcohol have not been successful, though it is believed that alcohol may be an accessory factor in the production of cirrhosis due to bacterial infection. In attempting to produce experimental cirrhosis by the use of agents which might possibly exist as

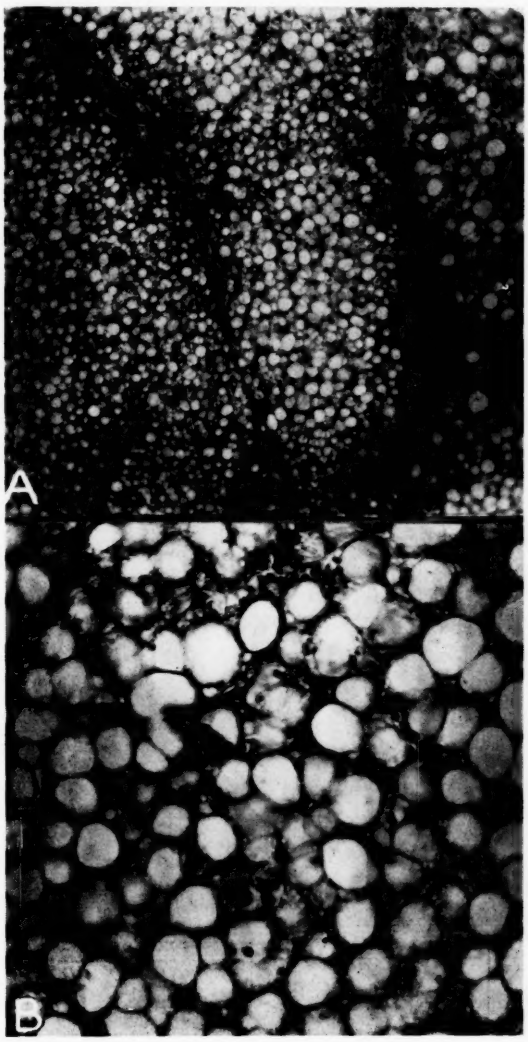

Fig. 1.—Photomicrograph shows (*A*) low-power field from a hypertrophic fatty liver. Note abundance of fat and very narrow periportal septa. *B*, High-power magnification. The liver cells contain large fat droplets and the nuclei are displaced to the periphery.

impurities in alcoholic beverages, Mallory was successful only with the use of copper. The recent work of Connor[7, 8] on the development of fatty livers in chronic alcoholism and the development of cirrhosis in fatty livers is interesting. Following the work of Himwick and Fazekas on liver metabolism in diabetes, Connor is of the opinion that in alcoholics there is a similar deficient carbohydrate

utilization. Because of the action of alcohol as a cell and tissue toxin there is a definite deficiency in carbohydrate metabolism and fat oxidation. In starvation or a carbohydrate-poor diet, such as is common in many alcoholics, sugar is withheld from the metabolic cycle. It has been shown that in experimental diabetes, in starvation, and in alcoholic intoxication the respiratory quotient of the liver is lower than that of the body as a whole or when fat alone is being consumed. In alcoholics it is probable that a condition similar to that of diabetes is produced and because of insufficiently utilized carbohydrates there is a piling up of unoxidizable fat within the liver cell.

The development of cirrhosis in a fatty liver, Connor states, is the result of pressure of the liver cells distended with fat upon the sinusoids. The sinusoids become collapsed and a thickening of the wall of the sinusoids is an early feature. The thickening of the walls with the formation of collagenous material promotes a reticulum production about the liver cells which have degenerated as a result of anoxemia. Fibroblastic proliferation follows. The interlobular stroma likewise is often stimulated to proliferate by the mechanical pressure of the distended liver lobules. Connor believes that the transition from a fatty liver to one of cirrhosis with very little or no fat is a result of the decreased carbohydrate intake an alcoholic assumes when his liver begins to provoke gastrointestinal symptoms.

Our material consists of the necropsy records of 356 cases of fatal alcoholic cirrhosis occurring in 12,267 consecutive autopsies performed at the Cook County Hospital from January 1, 1929, to July 1, 1939, inclusive.

Incidence.—There were 356 cases of cirrhosis, constituting 2.83 per cent of all our cases. This corresponds to Mallory's report of 2.2 per cent, but is higher than the incidence reported by Menne and Johnson,[9] or Evans and Gray.[10] It is somewhat lower than the incidence in Japan reported by Suzuki.[11] The yearly variation of cirrhosis of the liver, we believe, is significant (see Table I). Starting from a high level for 1929, there is a steady drop, reaching a low point in 1932. From 1933 there is a steady staggered rise. Though we are very reluctant to conclude from post-mortem records that a disease which may last ten or twenty years before resulting in death was less prevalent during a seven-year period, we cannot help noticing the regularity of the curve of yearly incidence. It can hardly be a coincidence that the curve is downhill until 1932 and has

TABLE I

INCIDENCE OF CIRRHOSIS

YEAR	AUTOPSIES	CIRRHOSIS	PER CENT
1929	955	28	2.93
1930	986	21	2.13
1931	1,114	25	2.24
1932	1,207	21	1.74
1933	1,192	31	2.60
1934	1,300	35	2.69
1935	1,176	40	3.40
1936	1,242	38	3.06
1937	1,167	52	4.45
1938	1,327	45	3.39
1939 (6 months)	601	20	3.35
Total	12,267	356	Avg. 2.83

been rising since. This peculiar level of incidence of a decrease until the year of the repeal of the prohibition amendment, followed by a steady rise, corresponds to the results of Evans and Gray[10] in their report of 17,874 autopsies at the Los Angeles General Hospital, and those of Boles and Clark[6] in their report of 4,000 autopsies at the Philadelphia General Hospital as well as the opinion of Rowntree,[12] who collected statistics from several institutions and public health agencies.

Sex.—Of our cases 244, or 68.5 per cent, were males, and 112, or 31.5 per cent, were females. Compared to our normal ratio of male to female autopsy material, which is two to one, we can conclude that cirrhosis occurs about twice as frequently in men as in women. These findings correspond with those of American observers, but are at odds with those of Japan and France. Suzuki,[11] in a report of 21 cases of cirrhosis out of 596 autopsies performed at the Kumamoto Medical College, found 19 males and 2 females. Paraf and Klotz[13] in a two-year period found that out of 1,030 females hospitalized at the Hôpital de Paris there were 23 cases of cirrhosis, or 2.2 per cent. In the same period, of 1,311 male admissions only 6, or 0.53 per cent, showed cirrhosis of the liver. The comparison, more than four times as frequent in females, is interesting and may open the way for a discussion on alcoholism in the French woman. The relation of sex to the occurrence of jaundice and ascites is noteworthy. The males in our group, though constituting 68.5 per cent of all our cases, comprised 76.4 per cent of all patients who had ascites alone. Conversely, the females comprised 39.5 per cent of all the cases having jaundice alone, somewhat above the figure for percentage of women having cirrhosis (see Table III).

Race.—There were 308, or 85.9 per cent, white and 50, or 41.1 per cent, colored persons in our series. Sex appeared to have no relation to race in our series, the male to female proportions being the same in both races. In our institutions where 35.8 per cent of our autopsy material consists of colored persons, the marked discrepancy of incidence is noteworthy.

TABLE II

DISTRIBUTION OF SEX AND RACE IN VARIOUS AGE GROUPS

	UNDER 30	31-40	41-50	51-60	61-	TOTAL
Male	1	22	68	89	64	244
Female	12	28	36	22	14	112
Colored	5	11	14	13	7	50
White	8	39	90	98	71	306
Total	13	50	104	111	78	356
Per cent	3.6	14.1	29.2	31.2	21.9	

Age.—Our youngest patient was 16 years; our oldest, 77 years. Thirteen, or 3.6 per cent, of our cases were under 30 years; 60, or 14.1 per cent, were 31 to 40; 104, or 29.2 per cent, were 41 to 50; 111, or 31.2 per cent, were 51 to 60; and 78, or 21.9 per cent, were over 60. The distribution of the various age groups in relation to sex and race can be seen in Table II. It appears that though 76, or 67 per cent, of the females were under 50, only 91, or 37 per cent, of the males were in the corresponding age group. The majority (64 per cent) of the males fall into the 41 to 60 age group while 57 per cent of the women are in the 31

to 50 age group. We believe that the increased incidence of cirrhosis in younger women in our series indicates mortality rather than pure incidence. Women with cirrhosis die at a younger age than do men. This is bolstered by the face that out of 13 cases in our youngest age group (under 30) 12 were women.

History of Alcoholism.—In 12 of our cases there was no clinical history available. Of the remaining 344 a definite admittance of chronic alcoholism was obtained in 144, or 41.9 per cent. We consider this to be far below the true incidence of indulgence in alcohol of the patients constituting our material. In obtaining the history of illness from the patient, the question of alcoholism is merely a part of the general survey of his habits. Unless a condition similar to cirrhosis or neuritis is suspected, the intern taking the history paid no more attention to the patient's temperance habits than he did to the amount of coffee or tea he drank or to the number of cigarettes he smoked. When a patient enters with obvious signs of intestinal obstruction, in diabetic coma, or with a perforated duodenal ulcer, as a great deal of our material does, the intern, with some justification, is not concerned with the patient's social habits and certainly does not anticipate the statistical survey which may result from his history. However, with the use of mimeographed forms for detailing a patient's history, our material is becoming more accurate and the only error remaining in a history of alcoholism will be that of the patient's reluctance in admitting it.

TABLE III

DISTRIBUTION OF JAUNDICE AND ASCITES

	JAUNDICE	ASCITES	JAUNDICE AND ASCITES	TOTAL JAUNDICE	NEITHER JAUNDICE NOR ASCITES
Male	26	65	87	113	66
Female	17	20	39	56	36
White	37	72	114	151	83
Colored	6	13	12	18	19
Under 30	4	1	3	7	5
31-40	8	7	21	29	14
41-50	13	21	46	59	24
51-60	16	31	35	51	29
Over 60	2	25	21	23	30
Total	43	85	126		102
Per cent	12.1	23.9	35.4		28.6

Jaundice and Ascites.—Jaundice occurred in 169, or 47.7 per cent, of our cases, and ascites in 211, or 59.2 per cent. The incidence of jaundice and ascites in relation to age, sex, and race is shown in Table III. It has already been pointed out that females are more likely to have jaundice without ascites and males are more likely to have ascites without jaundice. There are other significant facts apparent from Table III. Though the youngest age group constitute only 3.6 per cent of all the cases of cirrhosis, they comprise 9.3 per cent of all those having jaundice alone; the oldest age group, which make up 21.9 per cent of all our cases, constitute only 4.6 per cent of all cases of jaundice occurring alone. The incidence of jaundice alone shows a steady decrease as the age increases, while the occurrence of ascites alone increases with age. The combination forms (jaundice with ascites and neither jaundice nor ascites) seem to produce

opposing parabolic curves. Jaundice plus ascites is most prevalent in the middle age groups and is low in the extreme age groups; absence of both jaundice and ascites is lowest in the middle groups and highest in the extreme.

The ascites is produced by transudation of fluid from the mesenteric veins, a part of the portal system, as a result of obstruction and reduction in the size of the vascular bed. Our results of the occurrence of jaundice are at some variance with the common conception that jaundice does not occur frequently in this type of cirrhosis. Boyd says, in connection with the disturbed hepatic architecture seen, "Indeed it is to be wondered that jaundice is not a more marked feature in portal cirrhosis." Jaundice, with or without ascites, is most common in younger people. It occurred in 53.82 per cent of those under 30 years; 58 per cent of those 31 to 40; 56.7 per cent of those 41 to 50; 45.9 per cent of those 51 to 60; and 29.47 per cent of those over 60. We may interpret these results to indicate that jaundice is a late manifestation, late in the life of the disease rather than in the life of the patient. We may assume by the comparatively low incidence of jaundice in the older age group that these people survived because they did not develop jaundice, and that if they had developed jaundice at an early age they would have died at the earlier age to correspond with our increased incidence in the younger age groups.

Jaundice in cirrhosis is not due merely to compression of the bile ducts by connective tissue. It is probably due to the inability of the liver cells to excrete the bile pigment which is sent to the liver in normal amounts. The liver cells are abnormal, both early in the disease when the noxious agent which provokes connective tissue proliferation attacks them, and late in the disease when the increased connective tissue obstructs the sinusoids so as to produce venous stasis and subsequent anoxemia of the liver cells.

Esophageal Varices.—In our series there were 103 cases of esophageal varices constituting 28.6 per cent of the total (76 males; 27 females). Of these, in 33, or 32 per cent, of the number of cases with dilated and varicose esophageal veins, there were definite bleeding points noted. In many of these a massive hemorrhage was the immediate cause of death. This hardly corresponds with the report of McIndoe,[14] who notes a mortality due to hemorrhage of 50 per cent in 26 cases of advanced cirrhosis.

The incidence of esophageal varices in relation to age, as seen in Table IV, shows an increase with age until the 60-year-old group is reached, and is followed by a marked decrease. In examining the incidence of ruptured esophageal veins in the different sexes, we noted that though the females comprise 26 per cent of the total cases of varices and 20 per cent of those which have not ruptured, they constitute 39.3 per cent of the ruptured group. In addition, of the 27 women who had esophageal varices, 13, or 48.1 per cent, ruptured as compared to 20 of 76, or 26.3 per cent, of the men. This seems to indicate that though the presence of esophageal varices is somewhat less common in women than it is in men, when they do occur in women they are more likely to rupture.

Operations to produce more abundant anastomoses between the portal and systemic venous systems have been done repeatedly since 1889 when von der Meulen performed the first at the suggestion of Talma. After Morison performed the first successful one in 1895, varying reports of success have been

TABLE IV

ESOPHAGEAL VARICES IN RELATION TO SEX AND AGE

AGE	VARICES	MALE	FEMALE	TOTALS	
Under 30	Unruptured	0	1	1	3
	Ruptured	0	2	2	
31-40	Unruptured	6	1	7	13
	Ruptured	1	5	6	
41-50	Unruptured	15	7	22	34
	Ruptured	7	5	12	
51-60	Unruptured	20	4	24	34
	Ruptured	9	1	10	
Over 60	Unruptured	15	1	16	19
	Ruptured	3	0	3	

reported by Monprofit, Bunge, White, Mayo, and Tempsky. In a recent paper Grinnell[15] lists 23 cases in which 22 were followed up after the performance of surgery. He reports 9 per cent symptom free after a period of years; 32 per cent improved and 59 per cent unimproved. With an operative mortality of 27 per cent these figures are not encouraging.

TABLE V

BLOOD PRESSURE AND WEIGHT OF HEART

AGE	SYSTOLIC BLOOD PRESSURE					WEIGHT OF HEART		
	UNDER 120	120-140	140-160	160-180	OVER 180	UNDER 325 GM.	325-400 GM.	OVER 400 GM.
Under 30	4	4				13	0	0
31-40	12	11	7	2	4	23	10	11
41-50	28	23	20	4	4	48	35	21
51-60	29	16	25	15	7	37	29	31
Over 60	13	18	11	9	7	29	17	25
Total	86	72	63	30	22	150	91	88

Blood Pressure.—It is often stated that a persistently low arterial blood pressure is maintained in cirrhosis of the liver. Our material included cases of hypertension as well as those of hypotension. Of 273 cases in which a record of the blood pressure was available there were 158 in which the systolic arterial pressure was under 140 and 115 in which it was above 140. Table V indicates the distribution of the arterial tension in relation to the age of the individual; the weight of the hearts, an increase which often corroborates the clinical finding of hypertension, is also shown. In 179 cases the heart showed anatomical evidences of hypertrophy. There seems to be no indication that cirrhosis of the liver per se has any marked effect on the cardiovascular system.

Weight of the Liver.—In the 343 cases we recorded, 214, or 62.4 per cent, might be termed "hypertrophic"; 129, or 37.6 per cent, were "atrophic," and 66, or 19.2 per cent, were of the hypertrophic fatty form. The comparison of the incidence of the various liver weights in each age group is interesting (see Table VI).

TABLE VI

WEIGHT OF THE LIVER

AGE	UNDER 1,000 GM.	1,000-1,500 GM.	1,500-3,000 GM.	OVER 3,000 GM.	TOTAL
Under 30	1	2	7	3	13
31-40	4	11	28	7	50
41-50	8	28	54	13	99
51-60	5	36	55	10	106
Over 60	13	25	34	3	75
Total	31	98	178	36	343

Weight of the Spleen.—In one of our series a splenectomy had been done long before admission to the hospital; in four others the weight of the spleen was not recorded. Of the recorded weights the smallest was 50 Gm. and the largest was 2,045 Gm. Except for 12 the spleens were unanimously enlarged. Table VII shows the weights of the spleen in relation to sex and race. There is no marked difference in the sexes. In the races, however, there is a great variation. In 12 of 50 colored persons (24 per cent) the spleen weighed less than 100 Gm. as compared to 4 per cent in white patients. Sixty-two per cent of the colored persons had spleens weighing less than 200 Gm., while in 52.6 per cent of the whites it weighed more than 300 Gm. It is well known that the normal weight of the spleen in colored persons is less than in white, the discrepancy, however, in our results can hardly be explained on that basis. The abnormal constitution of the red blood cell in the Negro race is shown in its peculiar tendency toward sickling. We can only infer, at this point, that the marked difference in the weights of the spleens in our colored patients as compared to that in the white is merely another manifestation of the constitutional difference in the hematopoietic tissues of the Negro race.

TABLE VII

WEIGHT OF THE SPLEEN

WEIGHT	UNDER 100	100-200	200-300	300-400	400-600	OVER 600	TOTAL
Colored	12	19	7	4	8	0	50
White	12	65	66	62	68	28	301
Male	12	56	53	47	53	20	241
Female	12	28	20	19	23	8	110
Total	24	84	73	66	76	28	351

Pancreas.—There was no marked variation in the weights of the pancreas except in isolated cases, one weighing as much as 230 Gm., another as little as 30 Gm. In 129, or 36.2 per cent, of our cases, a well-marked fibrosis was noted.

There were five cases in which calculi were found in the pancreatic duct. This pancreolithiasis may be considered secondary to the cirrhotic condition of the pancreas. Cirrhosis was noted in 50 per cent of all cases of pancreolithiasis encountered in our necroptic material.

Carcinoma of the Liver.—There were 11 cases, or 3.09 per cent, of primary hepatocellular carcinoma of the liver as an incidental finding in our material. In the same period, however, there were 30 cases of primary malignant hepatoma; 11, or 37 per cent, had an associated cirrhosis. Both types of carcinoma, the massive discrete form and the multicentric form, were encountered. The in-

cidence of primary carcinoma in cirrhosis (3.09 per cent) and that of cirrhosis in primary carcinoma (37 per cent), we feel, corroborates the well-established conception that cirrhosis is the primary condition and that the tumor probably arises from the hyperplastic nodules. Worthy of note at this point is the incidence of primary carcinoma of the liver. Smith[16] in 1933, using part of our autopsy material, found 25 cases in 4,044 autopsies, an incidence of 0.56 per cent. We have collected from our material 30 cases in 12,267 autopsies, an incidence of 0.24 per cent.

Pectoral Alopecia.—Among numerous signs and symptoms connected with cirrhosis of the liver, two findings have received little attention: gynecomastia[17] and loss of axillary hair.[18] At the Cook County Hospital during the past ten years absence of hair on the chest in cases of cirrhosis has been emphasized. There has never been any formal effort made to corroborate this finding.

Of the 244 males in this series the presence or absence of hair on the chest was mentioned in necropsy protocols in 89. Seventy-six of these had no hair and 13 had sufficient hair to prompt such descriptions as "moderate amount of hair," "there is a coarse hairy growth," and "abundant hair on the chest." The interpretation of these statistics is variable. If we assume that the prosector, when doing the autopsy, mentioned the hair on the chest only when he happened to think of it, the incidence of pectoral alopecia is 76 out of 89 cases, or 85.2 per cent. If, on the other hand, the prosector mentioned the hair only when it was absent the incidence is 76 out of 244 cases, or 31 per cent. If the latter is true, it is difficult to explain the 13 cases in which the presence of hair was definitely noted.

SUMMARY

Three hundred and fifty-six fatal cases of cirrhosis of the liver were selected from 12,267 necropsies for study. In 184 cases cirrhosis was the primary anatomic cause of death, while in 172 cases the cirrhosis was an incidental finding. The cases were classified into two main groups: (1) periportal cirrhosis (atrophic or hypertrophic forms), and (2) fatty cirrhosis (hypertrophic form with a large amount of fat and little or no fibrosis). A total of 62.4 per cent of the livers were enlarged and 37.6 per cent were atrophic. Jaundice and hepatorenal decompensation were a frequent finding in the second group of cases (hypertrophic fatty form).

There were 68.5 per cent males and 31.5 per cent females. As to race, 85.9 per cent were white and 14.1 per cent were colored. Jaundice occurred in 169 cases, or 47.7 per cent, and ascites was present in 211 cases, or 59.2 per cent.

As to age, 104, or 29.2 per cent, occurred between 41 and 50, and 111, or 31.2 per cent, between 51 and 60 years. Hemorrhage from esophageal varices was noted in 33, or 9.2 per cent of the cases.

Hypertension was noted clinically in 32 per cent of the cases. However, a higher incidence existed (50.3 per cent) anatomically, considering hypertrophy of the heart as an index of a pre-existent essential hypertension.

Splenomegaly was a constant finding.

The pancreas was fibrosed in 36.2 per cent of the cases and in 5 cases concretions in the pancreatic ducts were found. Cirrhosis was noted in 50 per cent of all cases of pancreolithiasis.

Pectoral alopecia was observed at necropsy in 37 per cent of the males, a finding often of clinical diagnostic aid.

Hepatocellular carcinoma was noted as an incidental finding in 3.09 per cent of the cases, while 37 per cent of the cases of carcinoma of the liver showed cirrhosis.

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SEX HORMONE ASSAYS IN THE MENOPAUSE THEIR CLINICAL SIGNIFICANCE*

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THE purpose of this communication is to report the results and possible clinical significance of estrogenic and 17-ketosteroid urinary assays of 28 women suffering from the "menopausal syndrome."

SUBJECTS

All patients presented some well-known signs and symptoms of the menopause. For purposes of study they were classified as: 1. Those patients that had either natural or artificial menopause. 2. Those with or without one of the most subjective of all menopause symptoms—"hot flashes."

PROCEDURE

At least two, and in some instances four, complete twenty-four-hour urine specimens were obtained on consecutive or alternate days until consistent daily estrogenic and androgenic values were obtained.

The urines were hydrolyzed and extracted by the Hershberg and Wolfe[1] method and were fractionated by the method of Gallagher and others.[2] Specimens were assayed for estrogenic values by injection in divided doses of an aqueous suspension into 15 ovariectomized mice. Values were reported in terms of gammas of estrone based on a standard dosage-response curve. 17-Ketosteroid assays were done by the Friedgood-Whidden[3] modification of the Zimmerman method, using a Cenco spectrophotelometer and reported as milligrams of androsterone.

Table I

	NO. CASES	AVG. AGE (YEARS)	MEAN ESTIMATE*	RANGE ESTIMATE	MEAN ANDROSTERONE†	RANGE ANDROSTERONE
Natural menopause	16	51	6.8	1.1-22.9	9.59	3.7-20.8
Artificial menopause	12	46.8	6.75	2.9-15.0	8.75	4.4-14.25
Menopause with flashes	13	48.5	5.61	1.1-15.0	8.75	4.4-14.25
Menopause without flashes	15	49.6	7.68	1.5-22.9	9.58	3.7-20.8

*Gammas estrone.
†Milligrams androsterone.

DISCUSSION

Table I shows that there is a relationship between the mean values of the female and male sex hormone and symptoms; e.g., patients that did have "hot

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flashes'' had lower mean estrogenic values than those that did not exhibit this phenomena. This is not in accord with the observations of Fluhmann and Murphy,[4] who found there was no correlation between symptoms and the estrone content of the blood in the menopause patients.

Other interesting observations during this study were:

1. Some patients with severe menopausal symptoms were found to have female sex hormone values considerably above our normal values in the presence of a normal amount* of male sex hormone (Case 1).

2. Other patients had male sex hormones in the lower limits of normal and female sex hormones at the upper limits of normal.

3. Certain patients had male sex hormones at the upper limits of normal and female sex hormones at the lower limits of normal.

Our observations during this study: the fact that Salmon,[5] and Shorr et al.[6, 7] have shown that testosterone propionate in large doses can relieve menopausal symptoms and that all clinicians have observed certain menopausal patients who will not respond to adequate estrogenic therapy, suggests a theory that possibly the menopausal syndrome may be due to:

A. A lack of female sex hormone (Case 2).

B. A lack of male sex hormone (Case 1).

C. Sex hormone imbalance within normal limits, e.g., (1) Low normal male, high normal female (Case 3); or (2) high normal male and low normal female sex hormone (Case 4).

Several other interesting cases which will be reported in detail later were observed. One woman, who five years previously had had a complete hysterectomy and ovariectomy by a competent surgeon, excreted estrone in amounts far above the upper limit of normal. Another patient who had normal amounts of estrone and androsterone was found to have a vaginal smear indicative of marked estrogenic deficiency. She was given adequate estrogenic therapy by her local physician until a normal vaginal smear was attained with no clinical improvement in symptoms.

TREATMENT

The results of the urinary assays were used empirically to guide hormonal treatment. Patients in group A were given female sex hormone. Patients in group B were given male sex hormone. Patients in group C_1 were given male sex hormone and those in group C_2 were given female sex hormone. Some patients with normal female and male sex hormones were given female sex hormone and served as controls (Case 5). Space does not permit publication of all case histories, but the following are examples of types of cases observed:

CASE 1.—Female, aged 40 years. Complaints: Marked nervousness, headache, excitability, fatigue, and loss of libido following an artificial menopause three years previously, with an average excretion of 22.9 gammas of estrone and 8.1 mg. of 17-ketosteroids in twenty-four hours.

Treatment: Testosterone propionate was given in two courses of 10 mg. every four days for six weeks, with a rest period of two weeks in between courses.

*On a basis of as yet unpublished observations by Samuels, Yolton, and Winther at the University of Minnesota, arbitrary normal values for female sex hormones were assumed to be from 2.5 to 7.5 gammas of estrone and from 5 to 15 mg. of androsterone per twenty-four-hour specimen at the midmenstrual period. Thus, in those menopausal patients still menstruating specimens were collected at the middle of the cycle.

Results: Decided improvement in stability of equilibrium, loss of headache, definite improvement in fatigue, and a gain of 15 pounds in weight.

CASE 2.—Patient aged 51 years. Complaints: Hot flashes, sinking sensations, excess perspiration, feeling of intense heat, and irregular menses. Average excretion of 2.25 gammas of estrone and 7.59 mg. of 17-ketosteroids in twenty-four hours.

Treatment: Progynon 10,000 units intramuscularly was given three times a week for two weeks, and two times a week for the next two weeks.

Results: Flashes practically disappeared as did most of the subjective symptoms.

CASE 3.—Patient aged 50 years. Complaints: Occasional hot flashes, marked periodic "migraine" headache, beginning shortly after cessation of menses at age 45. Stiffness of hands, back, and knees. Average excretion 6.6 gammas of estrone and 4.4 mg. of 17-ketosteroids in twenty-four hours.

Treatment: Two courses of testosterone proprionate, 25 mg. every five days for six doses, with a two weeks' rest period between courses. After this therapy the patient has been able to maintain good health with 10 mg. every twelve days.

Results: Marked diminution in joint pain and almost complete relief from headaches.

CASE 4.—Patient aged 48 years. Complaints: Artificial menopause of two years' duration, moderate depression, loss of libido, hot flashes, fatigue, and emotional instability. Average excretion 4.35 gammas of estrone and 14.25 mg. of 17-ketosteroids.

Treatment: Ethinyl estradiol (Schering) in doses of 0.15 mg. a day.

Results: Improvement of emotional instability and depression, increased sense of well-being, and reduction of hot flashes from ten to one daily.

CASE 5.—Patient aged 45 years. Complaints: Hot flashes, excessive perspiration, increased nervousness, irritability, and fatigue following hysterectomy and partial ovariectomy three years previously. Average excretion 4.1 gammas of estrone and 5.35 mg. of 17-ketosteroids in twenty-four hours.

Treatment: Patient insisted on hormonal therapy though there was no indication of a deficiency as judged by urinary assay.

Results: No improvement.

Case 1 demonstrates a patient with a comparatively low male, high female sex hormone ratio. *Case 2* is a patient with a deficiency of female sex hormone and adequate male sex hormone. *Case 3* shows a patient with adequate female sex hormone but a male sex hormone which is below normal. *Case 4* is a patient with a high male, low female sex hormone. *Case 5* is an example of one of our control patients with normal male and female sex hormones, adequately treated with female sex hormone with no results.

The results of treatment carried on during the seven months of this study, using urine assays as an empirical guide to therapy, have been gratifying in that treatment has been successful in most instances, and it is felt that the sex hormone assay has pointed the way to successful treatment of some patients and prevented the needless expense of therapy in others who need psychiatric rather than endocrine management.

SUMMARY

Routine determinations of the male and female sex hormones in the urines of menopausal patients may have clinical application in that (a) it may indicate whether male or female sex hormone therapy is needed; (b) aid in explaining a lack of response of certain menopausal patients to the usual estrogenic hormone therapy.

A disproportionate amount of either male or female sex hormones may in some patients cause certain menopausal symptoms which may be alleviated by proper endocrine therapy.

A correlation between the incidence of definite "hot flashes" and the estrogenic and androgenic content of the urine appears to exist.

Estrone has been found in the urines of patients who have had their ovaries and uterus removed.

Correlation of urinary sex hormone assays with clinical results in treatment of the menopause is indicated.

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A NOTE ON THE BLOOD GUANIDINE LEVEL IN MIGRAINE SUBJECTS*

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SINCE migraine headache is held by some authorities to be related to essential epilepsy, it was of interest to determine whether changes in blood guanidine occur in connection with migraine attacks, as Murray and Hoffmann[1] have found to be the case in connection with epileptic seizures. These latter reported that the concentration of guanidine-like substances in the blood of patients with essential epilepsy rose considerably during the aura preceding seizures, reached a high level during the convulsion, and returned to the basic level within an hour thereafter. It was also of interest to check by our technique the basal blood guanidine levels in epileptics and the levels in a group of patients of mixed pathology. A group of normals was tested as a control.

Table I shows the results obtained in this study. Differences between the averages for the groups tested were not significant. Migraine patients showed no

*From the Institute of the Pennsylvania Hospital, Philadelphia, and the Department of Psychiatry of the Woman's Medical College of Pennsylvania.
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TABLE I

BLOOD GUANIDINE-LIKE SUBSTANCES

Milligrams per 100 c.c.

NORMAL SUBJECTS	MIGRAINE PATIENTS*		EPILEPTICS BASAL	OTHER PATIENTS	
	BASAL	DURING ATTACK			
0.40	0.41	0.40	0.50	0.48	Depressed
0.47	0.45	0.54	0.43	0.44	Depressed
0.48	0.47	0.54	0.35	0.37	Depressed
0.50	0.51	0.40	0.41	0.40	Anxiety
0.40	0.40	0.51	0.40	0.53	Narcolepsy
0.48	0.30	0.60		0.33	Hypothyroid
0.48	0.61	0.49		0.40	Histamine headache
0.42	0.51	0.55			
Averages					
0.45	0.46	0.50	0.42	0.42	

*The figures under "basal" and "during attack" were obtained on different patients.

material change in blood guanidine in connection with attacks, and the basal levels in epileptics were normal, as were the levels in the group of mixed pathology.

By comparison of their figures for epileptics with those reported for normal subjects by Andes and Myers[2] (0.21-0.28 mg. per 100 c.c.), Murray and Hoffmann concluded that the basal levels in the epileptics studied by them were higher than normal. Our basal values for epileptics are approximately the same as Murray and Hoffmann's and do not differ materially from our figures for normals. They are higher than the normal level found by Andes and Myers. This discrepancy is possibly caused by the differences in technique used in measuring the color developed in the determination. The photoelectric method used by Murray and Hoffmann and ourselves may give higher values than the ocular method used by Andes and Myers.

The groups studied were composed of about equal numbers of men and women. The persons with migraine, all of whom suffered from typical hemicrania of long standing, were patients of the Migraine Clinic of the Institute of the Pennsylvania Hospital, or were persons connected in some capacity with the Institute. The epileptics were patients resident in the Pennsylvania Hospital, Department for Mental and Nervous Diseases, or in the Philadelphia General Hospital. The normal subjects were obtained from the medical, laboratory, and nursing staffs of the Institute. The group of mixed pathology were from the private practice of one of us.

Blood guanidine derivatives were determined in duplicate by the method of Andes and Myers, except that the color was finally developed in a total of 5 c.c. instead of 2.5 c.c. and was measured by the Klett-Summerson photoelectric colorimeter, using a No. 540 filter.

SUMMARY

1. No sign of abnormality or material change was found in the concentration of guanidine derivatives in the blood of migraine patients previous to or during attacks.

2. The basal level of guanidine-like substances in a number of epileptics was not higher than the level in normal subjects.

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THE APPARENT FRUCTOSE CONTENT OF HUMAN EXTRACELLULAR FLUID*

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IN EARLIER publications[1-3] the presence in human spinal fluid of fructose, or of a compound with properties closely resembling those of fructose, has been reported. This substance was found in much greater concentration in spinal fluid than in blood. It has seemed desirable to study human extracellular fluid obtained from sources other than the spinal canal to determine whether this rather unexpected finding is a general or a specific one. In the present note the results of a series of such studies are presented.

The material used in the present study was obtained from medical wards of the Buffalo General Hospital and the Edward J. Meyer Memorial Hospital. A few analyses were made upon specimens obtained at post-mortem examinations made by the department of pathology of the Buffalo General Hospital. Since edema fluid approximates cerebrospinal fluid in its chemical composition, several samples of this material were studied. These were obtained by inserting small metal tubes—the so-called "Southey tubes"—under the skin of patients having gross accumulations of fluid whenever it was possible to do so. It seemed desirable also to study fluids which were rich in protein. Specimens of this nature were obtained either at autopsy or by puncture and aspiration from various portions of the bodies of living patients. The patients for the most part were not fasting when the specimens were drawn. As far as could be determined all the specimens studied were sterile. Blood was taken at the same time as were the specimens of fluid.

The technical methods used were the same as those previously described.[2] Protein and probably other interfering compounds were removed with zinc sulfate and sodium hydroxide in the proportions recommended by Somogyi.[4] When the protein concentration was low, it was possible to prepare suitable filtrates with a minimum dilution of the initial material, but when blood was analyzed, or when the fluids studied were rich in protein, it was necessary to make dilutions which were frequently as great as 1 to 10. The concentrations of zinc and sodium hydroxide used were the same in all instances; only the relative volumes of the sample and of distilled water varied. Final analyses were made, as described in previous articles.[2] Reducing compounds were determined by the method of Folin and Wu,[5, 6] modified, when the concentrations were very

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low, according to the recommendations of Hubbard and Allison.[7] Fructose, and fructose-like compounds were determined by the resorcinol technique of Roe.[8] Glucose gives a color when treated with the reagents used by Roe, and it was, therefore, necessary to correct the values obtained for the error produced by the presence in all samples of relatively large amounts of this sugar. Two methods were used for this purpose during the study reported. In some instances the correction was based upon analyses of many solutions of pure glucose carried through Roe's technique. In other experiments an analysis was first made by the technique of Folin and Wu, and a solution was prepared which contained the same concentration of pure glucose as did the specimen of blood or fluid analyzed. This solution of glucose was carried through the procedure simultaneously with the analysis of the unknown solution, and read against a standard solution of pure fructose. By subtracting the value so obtained from the reading given by the sample, the corrected fructose content of the blood or fluid was determined. In a number of instances an attempt was made to determine the effect of a short period of fermentation upon the apparent sugar content of the fluids.[9-11] For this purpose suspensions of washed baker's yeast cells were added to the blood and fluid to give concentrations of 1 c.c. of cells in 5 c.c. of fluid. These stood half an hour to an hour at room temperature, and were then precipitated and analyzed as described above.

Table I contains the results found. Only the corrected values for fructose are given. Because the accuracy of the fructose determinations in fluid were much greater when the filtrate analyzed was only slightly diluted, the degree of dilution is given in each instance. The determinations of "fructose" content after fermentation are included, but the nonglucose reducing compounds, which appeared to add little to the value of the data, have been omitted. The average values of these substances were equivalent to 6 and 8 mg. of glucose per 100 c.c. of fluid and blood, respectively. In only one instance did a study appear unsatisfactory. Difficulty was experienced in removing protein from the abdominal fluid in Experiment 27, and it was necessary to reprecipitate with a second addition of zinc and sodium hydroxide in this instance. Whether this procedure affected the significance of the results could not be determined.

We believe that a number of interesting facts are shown by Table I. The average concentrations of glucose in the blood and fluid specimens were approximately the same—99.0 mg. per 100 c.c. in the former, and 103.7 mg. in the latter material. In a majority of the specimens, however, the concentration was higher in the extracellular fluid than in the blood. This difference was not due to the presence of nonfermentable reducing compounds in the specimens of fluid analyzed. It probably resulted from a number of causes. These include the relatively greater water content of the fluids, and the relatively low glycolysis which results from the low concentrations of cells which most of them contained. (Note that in the specimens of synovial fluid rich in cells the glucose content was unusually low.) It is possible also that an uneven distribution of sugar between the blood and the fluid, similar to that which has been reported in spinal fluid,[12] may have influenced the results, for most of the fluids were not taken in a postabsorptive state, and the findings may represent an equilibrium attained when the blood sugar concentration was high shortly after a meal.

TABLE I

TABLE I

NUMBER	TYPE OF FLUID	ANALYSES MADE BEFORE FERMENTATION					AFTER FERMENTATION		NOTES
		BLOOD		EXTRACELLULAR FLUIDS			BLOOD	FLUID	
		Glucose (mg./100 c.c.)	Fructose (mg./100 c.c.)	Dilution	Glucose (mg./100 c.c.)	Fructose (mg./100 c.c.)	"Fructose" (mg./100 c.c.)	"Fructose" (mg./100 c.c.)	
1	Edema	77	0.0	1:3.6	101	0.4	–	–	–
2	Edema	75	0.5	1:1.2	97	0.5	–	–	–
3	Ascitic	–	–	1:5	117	1.8	–	–	–
4	Pericardial	–	–	1:3	109	1.5	–	–	Obtained at post mortem
5	Pericardial	–	–	1:3	177	0.0	–	–	Obtained at post mortem
6	Pericardial	–	–	1:3	80	0.3	–	–	Obtained at post mortem
7	Ascitic	–	–	1:5	142	0.0	–	–	–
8	Ascitic	181	–	1:3	182	0.0	–	–	–
9	Edema	–	–	1:2	120	0.7	–	0.0	Same case as No. 10
10	Edema	–	–	1:3	93	0.5	–	–	Same case as No. 9
11	Pericardial	–	–	1:3	135	0.0	–	0.0	Obtained at post mortem
12	Pericardial	–	–	1:3	117	0.0	–	0.0	Obtained at post mortem
13	Edema	85	0.5	1:5	97	0.6	–	–	–
14	Pleural	87	0.8*	1:2	93	0.8*	0.0	0.0	–
15	Edema	99	2.8*	1:2	116	3.0*	1.5	1.8	–
16	Pleural	134	0.2*	1:2	160	0.2*	0.4	0.4	–
17	Edema	77	2.8*	1:2	98	2.7*	1.7	1.6	–
18	Pleural	112	0.9*	1:5	127	1.3*	–	1.5	–
19	Synovial	152	1.0*	1:10	67	0.3*	–	–	Rich in cells; same case as No. 23
20	Ascitic	85	1.2*	1:5	116	1.2*	–	–	–
21	Pericardial	108	0.2*	1:12	6	0.0*	–	–	Gross blood present
22	Edema	76	0.4*	1:2.4	92	0.2*	1.0	0.2	–
23	Synovial	95	1.5*	1:10	63	1.0*	0.2	0.8	Rich in cells; same case as No. 19
24	Ascitic	131	1.0*	1:10	131	1.0*	–	–	–
25	Ascitic	94	1.3*	1:10	115	0.0*	–	–	–
26	Pleural	117	3.0*	1:10	102	0.8*	–	–	–
27	Ascitic	108	0.9*	1:2.4	142	1.7*	0.6	0.5	Unsatisfactory filtrate (see text)
28	Pleural	108	1.0*	1:5	138	0.5*	0.0	0.6	Chyliform fluid
29	Edema	83	1.1*	1:10	63	0.0*	–	–	Fluid rich in protein
30	Edema	101	0.5*	1:2	110	0.2*	0.3	0.1	–
31	Edema	100	0.6*	1:2	110	0.4*	0.7	0.2	–
32	Edema	59	2.0*	1:2	85	1.3*	–	–	Same case as No. 33
33	Edema	53	0.3*	1:2	74	0.1*	–	–	Same case as No. 32

Under glucose are given the concentrations of total reducing compounds expressed as glucose.
Under fructose are given the concentrations of substances reacting with resorcinol expressed as fructose.
The filtrates prepared from blood represented a 1:10 dilution of the initial material.
Fructose values have been corrected for the reaction given by the glucose present. The correction used for the starred (*) values were based upon determinations run simultaneously with the analyses; for others a standard correction was determined in preliminary experiments.

Sugar may have been removed from the blood by active metabolic processes, and a complete re-establishment of conditions of equilibrium by diffusion not have been reached. It is interesting to note that in a series of analyses of blood and spinal fluid made by identical methods, glucose was regularly present in the spinal fluid in lower concentrations than in the blood.

The results of the fructose determinations were also interesting. The average value of the corrected "fructose" concentrations in all the 33 fluid specimens studied was 0.7 mg. per 100 c.c. This differs markedly from the results upon spinal fluid specimens previously reported, for the average found upon 32 specimens reported previously was 2.9 mg. per 100 c.c. The contrast between the individual specimens was as marked as was the difference between these averages. In only 8 fluid specimens was the concentration greater than 1 mg. per 100 c.c., while only four spinal fluid specimens contained concentrations which were less than this. Each of these four specimens was obtained from a patient with meningitis, and there seemed to be good reasons for associating the low values with the clinical condition of the patients.[3] In 26 of the 32 spinal fluid specimens the concentration of fructose was greater than 2 mg. per 100 c.c., while there were only two results in the present study which contained such amounts of fructose. It seems obvious, therefore, that there is a marked difference between the apparent fructose content of cerebrospinal fluid and other types of extracellular fluid.

The data were examined to determine whether there were any differences between values upon fluids obtained from different body cavities. As far as could be determined there were no such differences. The highest fructose concentration found was in a specimen of edema fluid, but another specimen apparently contained none of the sugar, and in several others the concentrations were of the order of 0.1 or 0.2 mg. per 100 c.c. As a comparison with peripheral edema fluid, which is almost regularly very low in protein, a number of specimens of pericardial fluid, in which the content is regularly high, were analyzed. The results of these analyses also covered a wide range, from 1.5 to 0.0 mg. per 100 c.c. The rather limited number of other types of fluid, chest fluid, abdominal fluid, and synovial fluid, all showed similar variations.

It seemed possible that the method of handling the specimens might have affected the results. As already stated, when the protein content was high, it was necessary to dilute the material in order to obtain a filtrate suitable for analysis; when the protein concentration was low, marked dilution was unnecessary. The average of the values obtained when specimens were diluted five or ten times was 0.65, and when diluted less than five times it was 0.76 mg. per 100 c.c. Such variations in technique, therefore, had no significant effect upon the results. It is certain, however, that the probable significance of any single figure obtained when the more concentrated filtrates were analyzed was greater than when dilution was necessary.

A comparison between the fructose content of blood and of the various fluids was made. In the 23 studies in which results of both determinations were available the average fructose content of blood was 1.1, and of the fluids, 0.8 mg. per 100 c.c. This relationship is quite different from that observed in the study of

spinal fluid, for in that investigation the average fluid content of fructose was approximately six times as great as was the concentration in blood. If the figures presented in Table I are examined in detail, it is evident that results upon individual subjects agree at least as closely together as do the average ones, for in only a third of the studies do the results differ by 0.5 mg. per 100 c.c. or more. In all but one of the analyses in which the differences were of this magnitude, both the blood and the fluid were diluted 1:10, and the errors of both determinations were great.

Because there was so little difference between the average blood and fluid values, it seemed worth while to calculate the correlation coefficient between the two sets of figures. This had been done in the study of spinal fluid, and no significant correlation was found. In this instance the coefficient was $+0.64 \pm 0.08$. These figures make it seem probable that the fructose, or fructose-like compound, found in the extracellular fluids is derived from the blood, or at least is in equilibrium with some similar materials in the blood.

In the discussion of the results obtained in the previous study[2] it was pointed out that it was possible that no fructose is present in blood, because the accuracy of the determinations is so low. It seems to us that the correlation which appears to exist between the fructose in blood and extracellular fluid is evidence of the actual existence of some similar compound in both media.

An attempt was made to determine whether the material giving the color with resorcinol was actually fructose. In 14 experiments the fluid was inoculated with massive suspensions of yeast cells, allowed to incubate, and the apparent fructose content was determined. The results could not be considered satisfactory. In all but 4 of these specimens there was some apparent destruction of "fructose," and in some instances the destruction was marked. There was frequently noted, however, a measurable color when the incubated material reacted with the resorcinol reagents. The results with blood were essentially similar. There are at least three possible explanations for this finding. It is possible that at least two different substances in blood and extracellular fluid react with the resorcinol reagent, and that only one of these is readily destroyed by such suspensions of baker's yeast as were used in these experiments. The second possibility is that some fructose may have escaped destruction by the yeast suspensions used. It was found that some yeast suspensions do not ferment fructose readily, and it is possible that such small amounts of fructose as were apparently present in these solutions may have escaped destruction. The third possible explanation of the results is an extraction from yeast cells of some compound which will react with the resorcinol reagent. A suspension of yeast cells in distilled water has been incubated and analyzed as a part of each study presented in the table. These control suspensions regularly showed no color upon analysis. It was found, however, that certain alkaline solutions do extract from yeast cells measurable amounts of some compound which gives the resorcinol reaction, and it does not seem impossible that the blood and fluids may have had a similar action upon the yeast cells. It is, therefore, impossible to decide the exact nature of the compound which gave the resorcinol reaction in the unfermented blood and fluid specimens. It may all have been fructose; it may have

been some compound which resembles fructose, but which is slowly fermented by or adsorbed upon yeast cells; there may have been two compounds present which react with the resorcinol reagent, only one of which is acted upon by suspensions of washed cells of baker's yeast.

CONCLUSION

Results of the determination of the apparent fructose content of abnormal human extracellular fluid obtained from various body cavities have been carried out. Traces of some compound giving a reaction with the resorcinol reagent were present in two-thirds of the specimens. The amounts were much smaller than those previously found in cerebrospinal fluid. They appeared to be derived from, or to be in approximate equilibrium with, similar substances in blood. Fermentation with resting yeast cells was carried out in an attempt to identify the compound definitely as fructose, but the identification was not made with certainty.

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LABORATORY METHODS

GENERAL

A STERILITY TEST FOR SULFANILAMIDE POWDERS*

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THE report of a death from tetanus traced to a contaminated sulfonamide powder[1] makes it evident that there is a present need for an adequate test to determine the sterility of these valuable chemotherapeutic agents. There would appear to be no question regarding the necessity of subjecting the powders to heat sterilization,[2, 3, 4] but there seems to be some controversy as to the amount of heat which can be applied without altering the drugs to such an extent as to render them useless and, in some cases, actually dangerous to administer.[5, 6] The margin of safety between the amount of heat necessary to kill any organisms present, and the amount which will yield a product unfit for use is comparatively small. It is therefore important to have available a test for the determination of the sterility of sulfonamide powders.

The procedure which has been used successfully in this laboratory is as follows: The contents of a shaker package (5 grams of sulfanilamide) are transferred aseptically to a flask containing 500 c.c. of sterile water. The flask is vigorously shaken for five minutes, allowed to stand for thirty minutes at room temperature, and shaken again for five minutes. The undissolved sulfanilamide is allowed to settle and 5 c.c. of the supernatant fluid are added to each of two tubes containing 20 c.c. of Brewer's medium.[7] The tubes are incubated at 37° C. for a period of seven days and examined for evidence of growth.

In the development of the above procedure it was necessary to select a medium capable of supporting both aerobic and anaerobic growth, and which would, in addition, nullify the bacteriostatic effect exerted by the sulfonamide under test.

Initial experiments demonstrated that Brewer's medium fulfilled these requirements. It contains a sufficient amount of sulfonamide-inhibitor to permit growth of three selected test organisms (*Staphylococcus aureus*–Pure Food and Drug strain, and Army Medical School strains of *Escherichia coli* and *Clostridium tetani*) in the presence of 50 mg. of sufanilamide. This amount of sulfanilamide is the maximum amount which could be present per tube of Brewer's medium in the execution of the aforegoing sterility test. Inasmuch as Brewer's medium, by assay,[8] contains 2 micrograms of p-aminobenzoic acid per 20 c.c., with the possible presence of additional sulfonamide-inhibitors, it

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seems to be adequate to neutralize the bacteriostatic action of the sulfanilamide under our test conditions. It was found that the addition of an excess of sulfonamide-inhibitor (50 micrograms p-aminobenzoic acid) offered no apparent advantage in the initiation of growth by a smaller number of organisms, although there was some evidence to show that the aerobic organisms grow out more rapidly in the presence of an excess of p-aminobenzoic acid. The test organisms, inoculated into tubes of sulfonamide-containing Brewer's medium in numbers ranging from 3 to 100,000 grew out well in 48 hours. The assembled data on recovery of test contaminants added to Brewer's medium containing sulfanilamide is given in Table I.

TABLE I

RECOVERY OF TEST CONTAMINANTS IN BREWER'S MEDIUM* IN THE PRESENCE OF SULFANILAMIDE

TEST ORGANISM	INOCULUM NO. CELLS	SULFANILAMIDE 50 MG./TUBE		SULFANILAMIDE 50 MG. + P-AMINOBENZOIC ACID 50 γ/TUBE		CONTROL NO SULFANILAMIDE	
		24 HR.	48 HR.	24 HR.	48 HR.	24 HR.	48 HR.
ESCHERICHIA COLI	10,000	–	++	++	++	++	++
	310	–	++	++	++	++	++
	32	–	++	++	++	++	++
	3	–	++	++	++	++	++
	0	–	–	–	–	–	–
STAPHYLOCOCCUS AUREUS	100,000	–	++	+	++	+	++
	10,000	–	++	+	++	+	++
	620	–	++	–	++	+	++
	60	–	++	–	++	+	++
	6	–	++	–	++	+	++
CLOSTRIDIUM TETANI	10,000	–	++	–	++	–	++
	1,200	–	++	–	++	–	++
	224	–	++	–	++	–	++
	23	–	++	–	++	–	++
	6	–	++	–	++	–	++

*20 c.c. volumes in 25 x 200 mm. tubes. ++ = heavy growth; + = moderate growth; – = no growth.

In order to determine the efficiency of the method in the recovery of a spore-bearing pathogen from sulfanilamide powder, samples were artificially contaminated with small numbers of *Cl. tetani*. Two packages of sterile sulfanilamide powder were contaminated with 20,000 and 450 cells of *Cl. tetani*, respectively. The powders were returned to their packages, sealed and allowed to remain at room temperature for two days. Contaminated and sterile sulfanilamide powders were then examined by the test procedure. No growth was observed in the sterile sulfanilamide controls, while tubes containing contaminated sulfanilamide powder showed excellent growth of *Cl. tetani* within 48 hours. It was further noted that when the flasks containing the suspension of the artificially contaminated sulfanilamide powders were allowed to stand at room temperature for 48 hours, recovery of the organisms was effected even though the sulfonamide powder was completely dissolved and the spores of *Cl. tetani* were necessarily exposed to a 1000 mg. per cent solution of the drug for at least a portion of that length of time. This is further evidence of the extreme resistance of *Cl. tetani* spores to high concentrations of sulfanilamide.

This procedure is applicable to other sulfonamides as well. It is necessary, however, first to determine the maximum quantity of drug which is inactivated by the sulfonamide-inhibitors present in Brewer's medium.

SUMMARY

A test for the determination of sterility in sulfanilamide powder is presented. It is simple in operation, and effective in the recovery of small numbers of spore-bearing pathogens from artificially contaminated sulfanilamide samples.

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A DEVICE FOR INJECTIONS INTO THE EAR OF RABBITS*

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WHEN a rabbit has to be injected again and again, difficulties arise as the principal veins become obliterated.

This difficulty can be overcome by a simple instrument. To the box (*a*) of an ordinary pocket lamp with an elevated convex lens (*b*) is attached a strong metal wire (*c*) of 4 mm. diameter, bent to correspond to the shape of the

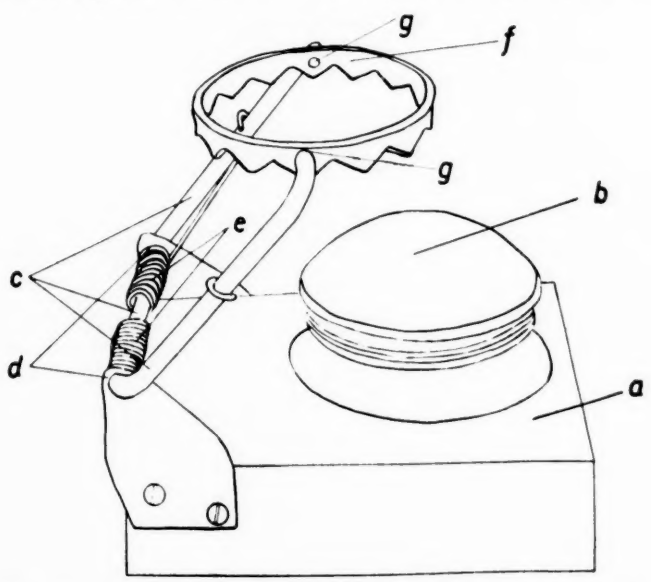

Fig. 1.—*a*, Battery casing; *b*, convex lens; *c*, metal wire; *d*, bearings; *e*, spring coils; *f*, serrated ring; and *g*, small holes in *f* into which the ends of *c* are inserted.

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lamp which runs in two bearings (*d*). The lower end of the wire is passed through two stiff spring coils (*e*) to give it the necessary pressure. At the upper end of the wire there is attached a serrated ring (*f*) which fits right over the convex lens of the lamp.

When using the instrument, the wire and the ring are lifted from the lens (as shown in the drawing), and the ear is interposed between lens and ring. The pressure of the springs fixes the ear with the teeth of the ring. Since the ring is serrated, the blood vessels of the ear under the teeth are compressed while there is free circulation in the vessels under the free spaces between the teeth.

The advantage of the convex lens is that it raises the ear of the rabbit above the ring so that the syringe can easily be directed to the vein to be injected without interference.

AN IMPROVED METHOD FOR DETERMINING THE SEDIMENTATION RATE OF THE RED BLOOD CORPUSCLES*

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ACCORDING to Reichel, the sedimentation rate is independent of the height of the blood column if the depth of the sedimentation does not exceed one-third the entire blood column. Also, it is independent of the volume of the test tube if the diameter of the latter is not smaller than 1.4 mm. With a narrower tube the capillary action would interfere perceptibly with the determination. The sedimentation rate also depends on the external temperature, being accelerated by heat and retarded by cold. In order to exclude this source of error—which in tropical countries shows its effect especially in summer, while in temperate zones it is more conspicuous in the winter—the following apparatus was constructed:

Apparatus.—This consists of a wide-necked thermos flask (see Fig. 1) fitted with an inner rack to hold the sedimentation pipettes. The stand is built to hold six sedimentation pipettes, springs keeping it at a given height. At the bottom of the stand six small test tubes with side apertures (2 to 3 mm. above their lower end), are fixed by means of small springs. The sedimentation pipettes are placed into these test tubes when a determination is being carried out.

Glass tubes (see Fig. 2) with a diameter of 1.45 to 1.6 mm., approximately 21 cm. long, the lower part without graduation, and cut flat. The ungraduated part corresponds to one-fourth the graduated part, i.e., from 30 to 40 mm. The upper graduated section is divided into centimeters, showing millimeter graduation as well. Length of graduated part is from 120 mm. to 160

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mm. Over the top a soft and transparent tubing of Para rubber is fitted, containing a movable glass bead.

Reagents.—1. 3.8 per cent neutral sodium citrate solution (tribasic). 2. Glycerol to moisten the tubing.

Procedure.—The pipettes should be rinsed with distilled water and dried with warm air only (no acetone or alcohol). Before blood is drawn from the finger tip the entire pipette should be rinsed with citrate solution. Then the citrate should be sucked up to the ungraduated part. This citrate is blown out into a small test tube. The blood is then allowed to flow (by capillary action) into the pipette—which should lie in as horizontal a position as possible—up to mark 0. Then let the blood flow into the citrate test tube, mix it well, but avoid formation of bubbles. The sedimentation test may be set up

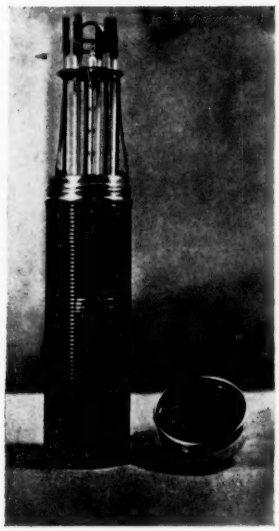

Fig. 1.

Fig. 2.

immediately or, at the latest, in the course of the next five hours. If the sedimentation is set up immediately, the pipette may be used again without being cleaned beforehand, otherwise it may be used only after thorough cleaning, drying, and renewed moistening with the citrate solution.

For setting up the reaction, moisten the tubing with a small drop of glycerol at its lower end. Slip almost the entire length of the rubber tubing (which is about 4 cm.) over the pipette. The glass bead pressed aside, the blood citrate mixture should be sucked up to mark 1. By drawing the rubber tubing upward, the blood column is raised up to mark 0. (If necessary

correct by shifting the bead inside the tubing.) Now the pipette is inserted into the stand which is put into the thermos flask, previously filled with water at 20° C. Readings are taken after thirty, sixty, and one hundred and twenty minutes with a slow sedimentation and after thirty and sixty minutes only if the sedimentation is fast.

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A SODIUM LAURYL SULFATE SOLUBILITY TEST FOR THE IDENTIFICATION OF PNEUMOCOCCI*

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SINCE Neufeld in 1900 demonstrated that pneumococci were soluble in bile, it has been customary to differentiate this group of bacteria from streptococci by treating the organisms with bile or one of the bile salt preparations. The difficulty in obtaining a clear specimen of bile is an obvious disadvantage when it is to be used for a test in which the final criterion is the clearing of a suspension of the organisms. Within recent years sodium desoxycholate has to a considerable extent replaced the use of bile. This bile salt gives a clear colorless solution, but it has the disadvantages of being expensive and, if the pH of the media is 6.6 or less, desoxycholic acid will precipitate and give a cloudy solution which obscures the test.

Lysis or clearing is considered by some investigators to be hastened by any process that kills the pneumococcus without impairing its enzyme activity (Mair, 1929). Bile, sodium desoxycholate, sodium ricinoleate, and most of the other substances which accomplish this are surface tension depressants. Because sodium lauryl sulfate possessed this property of lowering surface tension, it was thought that it might parallel bile in its action on pneumococci and streptococci. This compound was soluble in the presence of calcium and magnesium ions and in the presence of acid or alkali. With its low cost and the ease of obtaining a clear colorless solution, it would be more satisfactory than bile or sodium desoxycholate if the same results were obtained.

Bayliss (1937) studied the visible action of sodium lauryl sulfate on various microorganisms. Gram-negative organisms were usually cleared while gram-positive organisms remained turbid when broth cultures were treated with solutions of this compound. The pneumococcus was a notable exception to this rule. All available strains of pneumococci tested were found to be cleared in thirty minutes at 37° C. when the concentration of sodium lauryl sulfate was 0.2 per cent. All streptococci tested were found to be unaffected. The ease of obtaining

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satisfactory material for carrying out this test and of reading the results, because of the water clearness of the solution of sodium lauryl sulfate, made this procedure appear to be a desirable substitute for the bile solubility test. The cultures available at that time were limited. Since then the author has continued his observations on several hundred strains of pneumococci and streptococci.

EXPERIMENTAL

The action of sodium lauryl sulfate was studied by adding 0.5 c.c. of various concentrations of crude sodium lauryl sulfate* to an equal amount of a twenty-four-hour serum infusion broth culture of the organism. Controls of the culture plus saline, as well as uninoculated media plus sodium lauryl sulfate solution were made for comparison. After thorough shaking, the tubes were examined at frequent intervals and the degree of clearing was noted.

During the past five years several hundred pneumococcus cultures, including all the 32 types for which commercial typing antisera are available, have been subjected to the action of sodium lauryl sulfate. A few strains of pneumococci which did not fall in the types for which commercial typing antisera are available have also been tested. All freshly isolated strains have been readily soluble. Occasionally an old laboratory strain was encountered which was soluble with difficulty or, in rare instances, was unaffected. In such instances the effect on the organism of sodium desoxycholate or of bile was also proportionately decreased. Treatment of the pneumococcus in a manner which prevented the lytic action of bile also stopped any effect by sodium lauryl sulfate. Cultures heated to boiling for ten minutes were unaffected by either substance.

Pneumococci which have been recently isolated showed rapid clearing even in dilutions of sodium lauryl sulfate as high as 1:2,560. Table I illustrates the comparative clearing action of this compound and of sodium desoxycholate on type XI pneumococcus.

Numerous strains of pneumococci have given results comparable to those in Table I. With these results in mind a routine test for use in the identification of pneumococci was established. This consisted of adding 0.1 c.c. of a 2 per cent solution of sodium lauryl sulfate to 0.9 c.c. of a twenty-four-hour infusion broth culture. A control in which 0.1 c.c. of normal saline was added to 0.9 c.c. of the culture was also performed, although the results were striking enough so that this was not usually necessary. Both tubes were then incubated at 37° C. for thirty minutes before a final reading was made. With this routine procedure all freshly isolated strains of pneumococci were cleared.

A 2 per cent solution was best prepared by making 2 Gm. of crude sodium lauryl sulfate up to 100 c.c. with water. It was found desirable to heat, but

*"Dreft" is a sodium lauryl sulfate product manufactured by Procter and Gamble Company. It consists of about 50 per cent inorganic material, mainly sodium sulfate, and about 50 per cent organic compounds. Of the latter, about half is sodium lauryl sulfate and the remainder are compounds very similar in structure and properties, such as sodium sulfate esters of decyl, myristyl, cetyl, and oleyl alcohols. The normal variation of composition of the organic substances corresponds to that of coconut oil and palm kernel oil from natural sources.

"Dreft" is very soluble in water but frequently on standing at room temperature a white precipitate will settle out of the solution. This can easily be redissolved by warming. If kept at 37° C., the solution remains clear indefinitely.

McClure and Harris (1940) have recommended the use of a similar sodium lauryl sulfate product, Duponol WA, in solubility tests for pneumococci.

TABLE I

COMPARISON OF THE CLEARING ACTION OF SODIUM LAURYL SULFATE AND SODIUM DESOXYCHOLATE ON DIPLOCOCCUS PNEUMONIAE (TYPE XI)

CONCENTRATION	SODIUM LAURYL SULFATE					SODIUM DESOXYCHOLATE				
	TIME (MINUTES)					TIME (MINUTES)				
	1	3	5	10	30	1	3	5	10	30
1:20	1	2	3	4	4	2	3	4	4	4
1:40	1	2	3	4	4	2	3	4	4	4
1:80	1	2	4	4	4	2	2	4	4	4
1:160	2	3	4	4	4	2	2	4	4	4
1:320	2	3	4	4	4	2	2	3	4	4
1:640	2	3	4	4	4	2	2	2	3	4
1:1,280	1	2	4	4	4	-	1	2	2	3
1:2,560	1	2	3	4	4	-	-	-	1	2
1:5,120	-	1	1	2	3	-	-	-	-	1
1:10,240	-	-	-	-	1	-	-	-	-	-
1:20,480	-	-	-	-	-	-	-	-	-	-
Control	-	-	-	-	-	-	-	-	-	-

Key:
4, 90-100 per cent clearing.
3, 50-90 per cent clearing.
2, 25-50 per cent clearing.
1, 10-25 per cent clearing.
-, less than 10 per cent clearing.

not to autoclave, to aid in dissolving the compound. To remove any particles of dirt it was passed through filter paper. When kept at 37° C., it remained ready for use at any time.

Numerous strains of streptococci, including alpha, beta, and gamma types have been unaffected by the sodium lauryl sulfate solution. In each instance there has been either no clearing action or occasionally a slightly increased turbidity, possibly due to the increased dispersion of the intact organisms by this surface tension depressant. Parallel tests with bile and sodium desoxycholate have in each instance given similar results.

Microscopic examination at frequent intervals of the pneumococcus cultures following the addition of the sodium lauryl sulfate demonstrated decreasing numbers of intact organisms. With streptococci no disappearance of the bacteria was noted. Cultures of gram-negative bacteria to which relatively high concentrations (1:50 to 1:100) of the compound have been added usually showed macroscopic clearing, with a marked increase in the viscosity of the media, though microscopic examination revealed no marked lytic action on gram-negative organisms, such as *Escherichia coli*.

SUMMARY

Sodium lauryl sulfate rapidly cleared suspensions of pneumococci with disintegration of the organisms. Several hundred strains of pneumococci of various types have been studied and found to be lysed rapidly in a 0.2 per cent concentration of the compound.

All freshly isolated strains showed definite clearing within thirty minutes. Streptococci under the same conditions were unaffected.

The test is best performed by adding 0.1 c.c. of a 2 per cent solution of crude sodium lauryl sulfate ("Dreft") to 0.9 c.c. of a 24-hour culture. Usually

the results can be determined in a few minutes, but if clearing does not occur, it is necessary to incubate the mixture at 37° C. for one-half hour before the final reading.

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A METHOD FOR MAKING SECTIONS OF BONES AND TEETH*

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THE usual methods of studying the finer aspects of bone and tooth structure employ thin celloidin or paraffin sections, or slabs of bones or teeth sawed from the specimen and ground as thin as possible by some appropriate technique. The former method yields satisfactory studies of the cytologic picture, and the latter is capable of producing excellent studies of Haversian systems with their lamellae, lacunae, and canaliculi. Both of these methods are valuable and necessary, but both have certain drawbacks.

The method herein described, in supplementing the other methods, gives excellent pictures of bone architecture, with only the canaliculi showing poorly. The lamellae and lacunae, however, are demonstrated in a striking manner not seen in ground sections, or in the celloidin or paraffin sections. An additional advantage is obtained through the use of unstained and relatively thick (80μ to 120μ) sections, which offer a three-dimensional picture.

PROCEDURE

This method may be applied to bone in any state—fresh, fixed, or ordinary dried skeletal material. The best results are obtained when the bone is free from soft tissues and grease. Fresh bone may be fixed first or macerated in warm water until clean. Any appropriate fat solvent may be used for degreasing; acetone works well with fresh bone, and either acetone, benzene, or gasoline is satisfactory for dry bones.

Decalcification is accomplished in 5 per cent hydrochloric acid. The use of nitric acid is not recommended, since it seems to make the organic matrix brittle in some cases, and this results in a certain amount of splitting of the tissue. Hydrochloric acid decalcifies rapidly and does not cause maceration of the organic matrix if the tissue is not left in the reagent too long. Decalcification is considered to be complete when about 10 c.c. of the acid solu-

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tion covering the specimen is added to an equal volume of equal parts of 5 per cent ammonium hydroxide and 5 per cent ammonium oxalate, and no precipitate is formed. When decalcification is complete, the tissue is washed in tap water until the acid is removed.

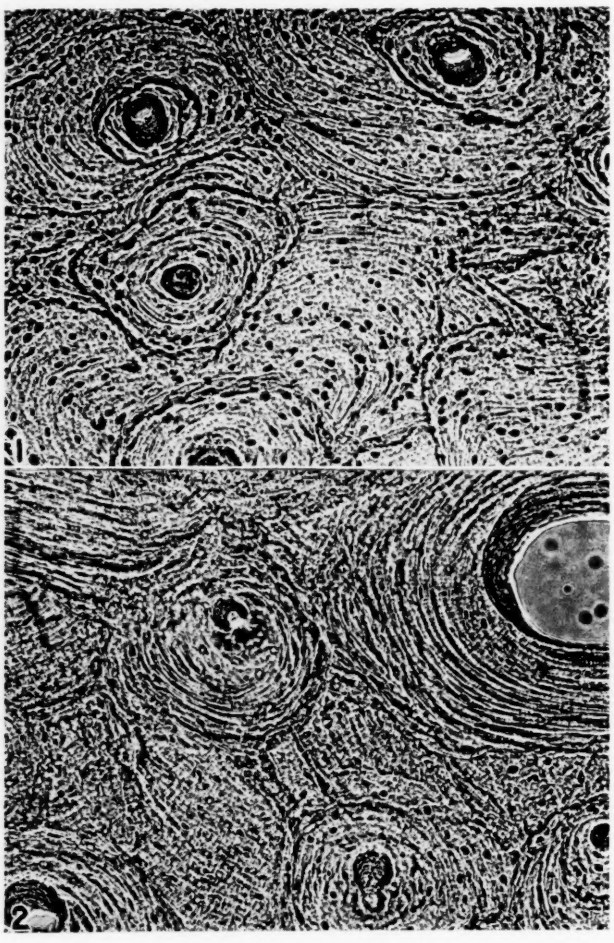

Fig. 1.—Section of a human metacarpal bone viewed with the substage condenser lowered and the concave mirror tilted to bring out the lamellar structure. The black dots are the lacunae (×160).

Fig. 2.—Another field of the same section as Fig. 1, showing the third dimensional effect obtained when focusing on a greatly enlarged Haversian canal. This section was cut freehand with a razor blade (×160).

The tissue next is placed in a potassium hydroxide-glycerin solution, such as is used in the Schultz technique for clearing embryos for demonstration of the skeleton. The formula used is:

Water	77 c.c.
Glycerin	20 c.c.
Potassium hydroxide	3 Gm.

The tissue remains in this solution until the matrix becomes semitransparent, when it is placed in rising grades of glycerin (50 per cent, 75 per cent, and 100 per cent) until completely cleared.

The glycerin is removed from the tissue by washing in several changes of 70 per cent alcohol. This is followed by replacing the 70 per cent ethyl alcohol with n-butyl alcohol, C.P. Two or three baths of butyl alcohol may be required in order that there may be no ethyl alcohol left in the tissue. The last butyl alcohol bath may be saved, and the alcohol may be used later to dissolve the paraffin out of the cut sections.

The next step is to infiltrate the specimen with paraffin and to embed for sectioning. At least three paraffin baths (one hour or longer in each bath—according to the size of the specimen) are recommended.

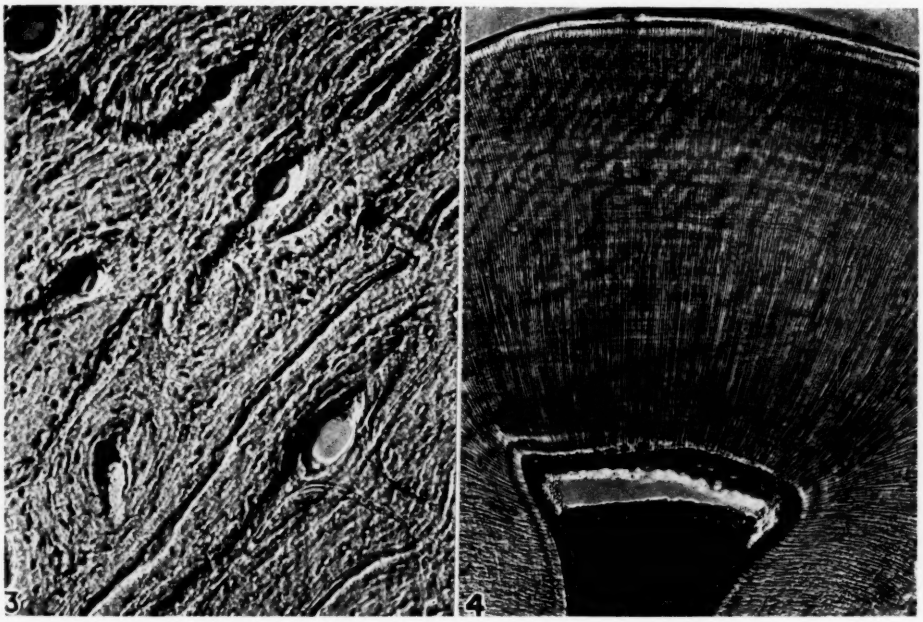

Fig. 3.—Section of the femur of a child about 8 years old. The substage condenser has been elevated as high as possible, and the concave mirror is tilted to give the impression of viewing the section by reflected light. Vascular channels are shown well, and the third dimensional effect is demonstrated. The black dots are lacunae. Section cut on microtome—120μ (×160).

Fig. 4.—Section of the tooth of a fish to illustrate the application of this method to the sectioning of teeth. Freehand section cut with a razor blade (×160).

Sections may be cut in any of three ways: (1) freehand with a razor blade; (2) with a well microtome and a straight-edge razor, or a microtome knife; or (3) with a celloidin microtome that will cut sections up to 200μ. The sections in Figs. 1, 2, and 4 were cut freehand. The section in Fig. 3 was cut at 120μ on a celloidin microtome. By cutting sections of different thicknesses, from 40μ to 200μ it appears that the sections from 80μ to 120μ give the best pictures of the arrangement of the circumferential lamellae, Haversian systems, interstitial lamellae, and the interrelationships of the Haversian canals.

Sections are put into a container with enough butyl alcohol to cover them. They remain rolled up until the butyl alcohol has dissolved away the paraffin in which they are embedded. Removal of the paraffin is accomplished

in the paraffin oven. The first butyl alcohol bath will remove most of the paraffin, but two more baths in butyl alcohol are necessary to remove all traces of paraffin. Glycerin is added to the butyl alcohol of the final bath in rising percentages—25 per cent, 50 per cent, and finally 100 per cent. Glycerin replaces the butyl alcohol and clears the sections.

The sections are mounted more or less permanently in glycerin jelly. They may be studied in glycerin, but need to be in a semisolid medium for photomicrography, or for handling over a period of time. Greater permanence may be given the slides by ringing the cover slip with gold size and Bell's cement. A thick jelly is recommended, according to the following formula:

U.S.P. granular gelatin	15 Gm.
Distilled water	50 c.c.
Glycerin	55 c.c.
Phenol crystals	2 Gm.

The gelatin is put into the water and melted with gentle heat. The glycerin may be added at this time after first dissolving the phenol in the glycerin. The melted glycerin jelly should be filtered through moist, hot flannel before using.

The sections are taken from pure glycerin and pressed between two filter papers to remove excess glycerin. The melted jelly is pipetted onto a warmed slide (the number of drops necessary is easily ascertained after several trials), and the section is placed in the jelly. The coverslip is applied, after warming over a flame, and the excess gelatin is pressed out from under the cover slip. Slides are readily cleaned after the gelatin hardens by cutting away any gelatin extending onto the slide beyond the cover slip. Wiping the slide with a damp cloth completes the cleaning process.

Proper use of the microscope in the study of the prepared slides is quite as important as the different steps of preparation of the tissue. The lighting effects that are desired to bring out the details of the sections, are obtained by manipulation of the substage condenser and mirror. In one position the section appears to be illuminated by reflected light, as seen in Fig. 3. Another position of the mirror and condenser gives the appearance of lighting by transmitted light, as in Figs. 1, 2, and 4. In the former position the surface topography of the section is seen in three dimensions, and by proper focus the details of the vascular channels and lacunae are emphasized. These details may be studied with various magnifications. In the latter position the details of lacunae and lamellae are emphasized. This brings out the true architecture of the bone—the boundaries of the Haversian systems and the relations of the interstitial and circumferential lamellae being displayed in a striking manner. To produce the effect of reflected light the condenser is raised as high as possible, and the mirror is tipped in different directions until the desired result is obtained. To bring out the lamellar structure the condenser is slowly lowered, and again the mirror is moved in different directions until the desired effect is obtained. The concave mirror is always used.

The use of the higher grades of ethyl alcohol for dehydration, and xylol, or other clearing reagents ordinarily used before passing the tissue into

paraffin, hardens the ostein, and changes it optically so that the structural details are shown very poorly, or not at all.

The use of Lendrum's technique (Carleton, 1938) for softening dense fibrous tissue was found to be advantageous with teeth and with bones larger than rat bones. The decalcified bone is placed in a solution of 4 per cent phenol for four to six days at room temperature. If any signs of maceration appear, the treatment must be stopped at once. The bone is washed in several changes of water at 45° C. until the odor of phenol no longer can be detected. The specimen is then cleared as described. The rest of the treatment is unchanged.

SUMMARY

Fresh, fixed, or dried bone specimens are decalcified in 5 per cent hydrochloric acid.

The decalcified bone is cleared in potassium hydroxide-glycerin solution, and finally in rising grades of glycerin (50 per cent, 75 per cent, and 100 per cent).

Glycerin is removed from the tissue by washing in 70 per cent alcohol, which is replaced by n-butyl alcohol (three baths).

The specimen is infiltrated with paraffin* and is embedded in a block of suitable size. Sections may be cut freehand with a razor blade; on a well microtome, with a section razor or microtome knife; or on a celloidin microtome. Thick sections are used for study, 80μ to 100μ.

Paraffin is dissolved out of the sections in several changes of n-butyl alcohol, and the sections are placed in several changes of rising grades of glycerin: 25 per cent, 50 per cent, and 100 per cent.

From 100 per cent glycerin the sections are mounted in a thick glycerin jelly, and are studied microscopically with different lighting effects attained by the raising and lowering of the substage condenser, and the movement of the concave mirror to reflect the light from different angles.

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Carleton, H. M.: Histological Technique, New York, 1938, Oxford University Press, page 39.

*Parowax produced by the Standard Oil Company of Ohio, Cleveland, Ohio, is used in this laboratory.

EYEGLASSES TO BE USED WITH THE BINOCULAR MICROSCOPE*

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FOR several years a pair of special eyeglasses has proved helpful to me, a hyperopic person, while using the binocular microscope. The glasses are crescent-shaped, 1.3 cm. high in the center. Over the upper concave border, I look into the oculars of the microscope. With the bitukni binocular one can sit in a very convenient position without strain on the muscles of the neck. (These eyepieces form an angle of 135° with the vertical tube.) I had about one-third of the upper rim of the oculars removed on the lathe, flush with the tube of the eyepiece, enabling me to get as near the eyepieces as can be done without glasses, and thus getting the benefit of the whole microscopic field. It also prevents the eyeglasses from catching at the microscope and it permits free movement of the head. When the upper portion of the eyepieces is cone-shaped, as in the Spencer outfit, this is unnecessary.

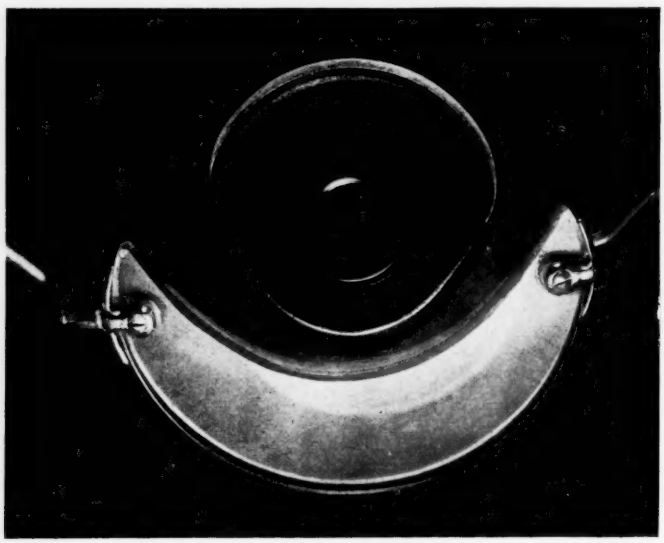

Fig. 1.

The glasses are weaker than the ones used for reading. Hence, without eye-strain and without much moving of the head, it becomes possible to overlook the table I am working on. At the same time, the glasses are strong enough for writing and reading. The strength of the glasses is a matter of individual choice. Except for continued reading and for observation of finer details, such glasses can be used for most laboratory activities. This obviates the too frequent changing of eyeglasses. Fig. 1 is self-explanatory.

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THE PITUITRIN CONCENTRATION TEST OF RENAL FUNCTION*

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POSTERIOR pituitary extract inhibits water diuresis by producing an increased reabsorption of water by the renal tubular epithelium. Its use, therefore, has been advocated as a substitute for the required period of water deprivation necessary in the performance of the standard Fishberg test. This period of water restriction represents a considerable inconvenience to the patient and a source of error when the patient fails to cooperate.

The use of pituitary preparations in the performance of kidney function tests was first advocated in 1930,[1] and since then sporadic references to its use have appeared in the literature.[2] Recently, Sodeman and Engelhardt[3] attested to the efficacy of surgical pituitrin in producing a urine, in 15 normal subjects, as concentrated as that produced by a "modified Fishberg technique."

The purpose of this study is (1) to compare, in unselected hospital cases, the urine concentrations obtained after an injection of surgical pituitrin without a period of water deprivation, with urine concentrations obtained in the same patient following the usual period of water restriction advocated by Fishberg; and (2) to judge the clinical applicability of the pituitrin test.

METHODS

Four procedures were employed:

1. Fishberg concentration test: The technique used was identical with that outlined by Fishberg.[4]
2. Pituitrin concentration test: Without fluid or food restriction the patient emptied his bladder at some designated hour, and 0.5 c.c. of surgical pituitrin (10 units) was injected subcutaneously. Three urine specimens were obtained hourly thereafter.
3. Volhard dilution test: 1,500 c.c. of fluid were administered orally within thirty minutes after emptying the bladder. Four hourly urine specimens were then collected.
4. Procedure 3 was repeated, but in addition 0.5 c.c. of surgical pituitrin (10 units) was injected after the ingestion of the fluid.

The specific gravity of the urine specimens obtained was measured by means of a clinical urinometer calibrated to a standard temperature. Corrections were made for temperature variations[5] and protein content[6] of the urine.

In 100 patients procedures 1 and 2 were carried out. In 25 of these all four procedures were performed.

For the purposes of this study a specific gravity of 1.020 was arbitrarily chosen to divide the cases into two groups. Those patients concentrating to 1.020

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or better in any single specimen were considered to have normal renal function, while those not reaching that standard were grouped as representing abnormal renal function. The pituitrin test was considered to duplicate the Fishberg test when it succeeded in producing a urine which fell into the same group classification as that obtained with the latter.

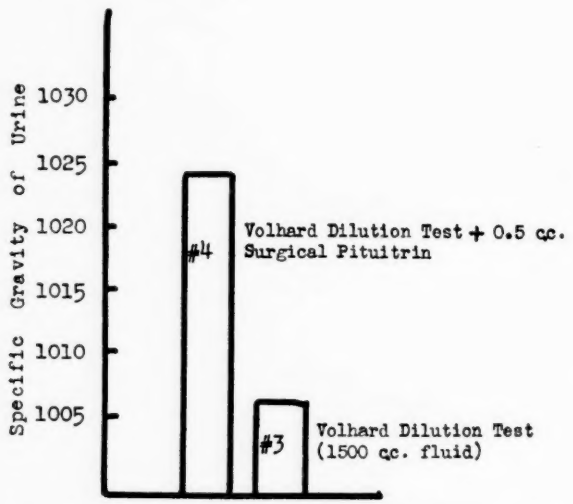

Fig. 1.—Average urine concentration produced by procedures 3 and 4.

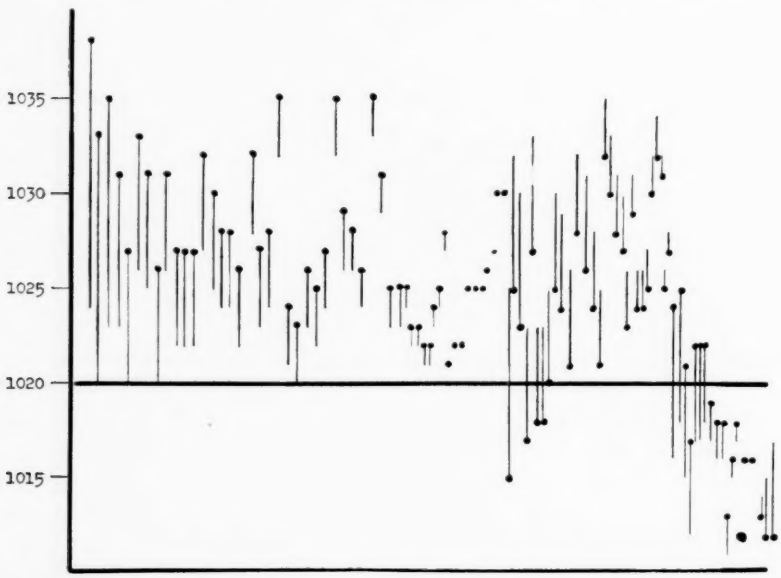

Fig. 2.—A comparison of Fishberg and pituitrin concentration tests of renal function in 100 patients.
• Pituitrin test (procedure[2])—highest concentration.
| Fishberg test (procedure[1])—highest concentration.

RESULTS

In 25 patients the Volhard dilution test (procedure 3) produced a dilute urine in contrast to the definitely more concentrated urine obtained when this

test was combined with the use of pituitrin (procedure 4). The average concentrations obtained are graphically reproduced in Fig. 1.

The pituitrin test compared favorably with the Fishberg test in 90 of 100 cases.

Of 81 patients, comprising a group concentrating to 1.020 or above by means of the Fishberg technique, 4 (4.9 per cent) failed to reach that specific gravity when the pituitrin technique was substituted. Of the 19 patients comprising the group with urine concentrations below 1.020 with the Fishberg technique, 6 (31.5 per cent) showed concentrations over 1.020 with the pituitrin test. These results are graphically represented in Fig. 2.

No deduction can be drawn as to the effect of pituitrin on urinary volume. While on the whole more concentrated urines were accompanied by a reduction in volume, examples were encountered in which alterations in specific gravity were independent of urinary volume.

DISCUSSION

The production of concentrated urines after the injection of 0.5 c.c. (10 units) of surgical pituitrin in the face of a quantity of ingested fluid that ordinarily would have produced a very dilute urine (procedure 3 and 4) definitely established the potency of the preparation of pituitrin used in these experiments.

It is apparent from the results obtained that the pituitrin test duplicated the Fishberg in 90 per cent of the cases investigated.

Of the 10 cases representing failure of correlation of the two tests, in only four did the pituitrin test fail to reach the high concentrations obtained with the Fishberg test. In the remaining six patients the pituitrin technique produced a sufficiently more concentrated urine to alter the estimation of kidney function from abnormal to normal. A clinical and laboratory evaluation of these six cases substantiated the interpretation derived from the pituitrin technique in that there was no history to suggest a latent nephritis, blood pressures were all within normal range, repeated urinalysis revealed no abnormal constituents, and the blood urea clearance tests in each instance were above 80 per cent of normal.

No untoward effects have been observed with the use of pituitrin even in severe hypertensives and in patients with coronary artery disease. We have avoided performing the test in pregnancy.

The technique of the test is simple, causes a minimum of inconvenience to the patient, and by eliminating the need for any specific preparation, permits the physician to exercise more complete control over the patient.

A statistical analysis of the 100 cases* revealed an average difference in specific gravity between the pituitrin and Fishberg tests of ±0.001, with a standard deviation of 0.0043. Thus, in any given instance within the statistical limits of ±2 times the standard deviation, the pituitrin test may yield a specific gravity of 0.009 more than, or 0.007 less than, the Fishberg test.

Since the normal kidney can produce specific gravities of greater variation than the abnormal, the cases falling below 1.020 were analyzed separately. These yielded an average difference of ±0.002, with a standard deviation of 0.0033.

*Martin S. Abel, M.D.

Thus, in any given instance within the statistical limits of ±2 times the standard deviation, the pituitrin test may yield a specific gravity of 0.008 more than, or 0.004 less than, the Fishberg.

Of the 100 cases, 47 had specific gravities ranging from 1.015 to 1.025. This significant intermediate zone revealed an average difference of –0.0002 and a standard deviation of 0.0029. Thus, values with the pituitrin test can vary from 0.005 more than, or 0.005 less than, the Fishberg in this group.

The analysis further indicates that any given pituitrin test yielding values of 1.025 or above, or 1.015 and below, will almost invariably agree with the Fishberg test. Within the range of 1.015 to 1.025 the probability of the two tests agreeing becomes less, diminishing as the arbitrary dividing line of 1.020 is approached.

However, the analysis presupposes that the Fishberg test is a constant standard and will not vary appreciably from day to day. We demonstrated this basic supposition to be invalid by a survey of 12 patients who revealed a significant day to day variation in specific gravities obtained with the Fishberg technique.

Before the pituitrin test may be accepted as a substitute for the Fishberg on the basis of its technical advantages and 90 per cent correlation revealed in this series, or rejected on the basis of its statistical probability of disagreement with the Fishberg test, one must substitute more substantial criteria for judging the clinical applicability of this new test. This can only come about in the future through long-range observation of patients in whom both tests have been performed.

SUMMARY

1. Surgical pituitrin in the dose used (0.5 c.c. or 10 units) is an active preparation capable of concentrating urine.

2. The substitution of pituitrin for the period of water deprivation used in the Fishberg test resulted in a 90 per cent correlation of the two tests.

3. Absolute failure of the test was encountered in only four cases.

4. In six cases the pituitrin test apparently more accurately reflected kidney function than the Fishberg test.

5. Statistical analysis of the results revealed an appreciable possibility of disagreement between the two tests when specific gravities range from 1.015 to 1.025.

6. A true clinical evaluation of the test will depend upon further comparative studies.

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CHEMICAL

THE STANDARDIZATION AND ASSAY OF HEPARIN BY THE TOLUIDINE BLUE AND AZURE A REACTIONS*

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WE BELIEVE that in view of the increasing use of heparin a direct chemical assay method is of decided advantage. In two previous papers[1, 2] the toluidine blue method of heparin assay (method I) was partially outlined. The present paper is a result of our efforts to increase the accuracy and to widen the scope of this method. In addition, since the biological assay methods for heparin differ greatly in results,[3] it seemed advisable to attempt the standardization of the unit of heparin by employing the colorimetric assay.

The toluidine blue reaction was described by Lison[4, 5] as specific only for sulfuric acid esters of high molecular weight, referring primarily to chondroitin sulfuric acid. Chondroitin sulfuric acid has the amino sugar galactosamine, whereas Jorpes and Bergstroem[6] demonstrated that heparin contains glucosamine. They identified heparin as a mucoitin polysulfuric ester and Jorpes[7] tested it with toluidine blue. Heparin was found to be a mucoitin sulfuric acid residue by Charles and Todd,[8] and these groupings were shown in the analytical values of the sodium salt by Reinert and Winterstein.[9] Barium heparin was shown by Charles and Todd[8] in a recent communication to vary in potency with the sulfur content.

Toluidine blue is not the only dye which gives a metachromatic reaction with sulfuric acid esters, for Conn[10] has shown that toluidine blue is very similar in composition to azure A. Therefore, we undertook to test azure A with regard to its relationship to heparin and in comparison with toluidine blue.

EXPERIMENTAL

We have used the following dyes manufactured by the National Aniline Chemical Company, toluidine blue under the name NU-3, and azure A under the name of NAz-7,‡ as certified by the Commission on Standardization of Biological Stains, Agricultural Experiment Station, Geneva, N. Y. Both products are on the market and, as Dr. Conn has been advised by the manufacturer, they have on hand enough NU-3 recently manufactured to last for about four years.§

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‡We are indebted to Dr. H. J. Conn for rechecking our sample NU-3 and for supplying us with the sample of Azure A (NAz-7).

§Personal communication, October 29, 1941.

We have been supplied with preparations of heparin by Connaught Laboratories, Lederle Laboratories, and Abbott Laboratories which were assayed biologically by Dr. A. F. Charles, Dr. C. M. Meadows, and Dr. W. C. Risser, respectively.

METHOD I

Procedure.—Make up 1:1,000 toluidine blue (TB) or azure A (AA) by adding 1.6949 Gm. TB (NU-3) to 1,000 c.c. or 1.1494 Gm. AA (NAz-7) to 1,000 c.c. of distilled water. In a series of test tubes place 1 u (Murray-Best unit*), 2 u, 3 u, 4 u, and 5 u of heparin CLT-110 (Connaught Laboratories, Toronto, 1 mg. containing 110 u), make up to 5 c.c. with distilled water, and add 1 c.c. of 1:1,000 toluidine blue (TB), 100 per cent dye strength. Shake, and after standing for fifteen minutes, centrifuge at 3,600 r.p.m. for fifteen minutes. The supernatant fluid is decanted and compared on the Duboscq colorimeter with a solution of 1:6,000 toluidine blue (100 per cent dye strength). The concentrations of dye are computed in gamma. Six cubic centimeters of the 1:6,000 toluidine blue solution contain 1,000 gamma of dye. It has been discovered by experience that those solutions containing from 300 gamma to 700 gamma give the most accurate comparisons. In view of this experience we select those concentrations between these limits for our computations and take the average as our result. After the residual dye concentrations have been computed, they are each substracted from 1,000 gamma (the original dye concentration), giving the amount of dye reacting with the heparin present. Dividing this quantity by the units of heparin in the test, the amount of dye reacting with each unit of heparin is obtained. Dividing the amount of dye reacting with each unit by 150 gives the strength in colorimetric (Copley-Whitney) units.

Results.—In the test on a Connaught Laboratories sample, 110 u per milligram (CLT-110), that we used as a standard, the following concentrations were determined: The 3 u test contained 585 gamma of dye and the 4 u test contained 431 gamma of dye after centrifugation. Subtracting these amounts from 1,000 gamma, the initial concentration, we obtain 415 gamma and 569 gamma, respectively, as the amounts of dye that reacted with 3 u and 4 u of heparin. Therefore, 415 gamma divided by 3 gives 138 gamma of dye reacting with each unit of heparin, and 569 gamma divided by 4 gives 142 gamma of dye reacting with each unit of heparin. The average of 138 gamma and 142 gamma is 140 gamma per unit of heparin. Since the valuation of the Connaught Laboratories appeared to us rather high, and in order to simplify future calculations and standardization, we decided to establish 150 gamma of dye per unit of heparin as the standard. Samples were diluted up to 1 u (Copley-Whitney) per cubic centimeter and tested as in the preceding method.

Three and 4 colorimetric unit tests diluted 1:5 and 1:10 were compared on the K & E spectrophotometer (Fig. 1). Samples of TB 1:10,909 and 1:15,000, diluted 1:5 and 1:10 and compared on the K & E spectrophotometer, were found to approximate the 3 unit and 4 unit tests, respectively. From the above comparisons, 0.15 mg. of 100 per cent TB—TB (NU-3) 59 per cent dye strength as TB; AA (NAz-7) 87 per cent dye strength as TB—were

*Murray and Best[11] fixed the unit of heparin as 0.01 mg. of a crystalline barium salt. The unit of the "pure" sodium heparin, which is based upon the barium salt, equals $\frac{1}{110}$ mg.

absorbed by each 1 u of heparin. On comparing subsequent tests on dilute solutions of heparin with standard TB and AA solutions of 1:10,909 = 3 u and 1:15,000 = 4 u in the Duboseq colorimeter, the accuracy was within less than 1 u of heparin provided the heparin solution as stock sample contained less than 30 u per cubic centimeter.

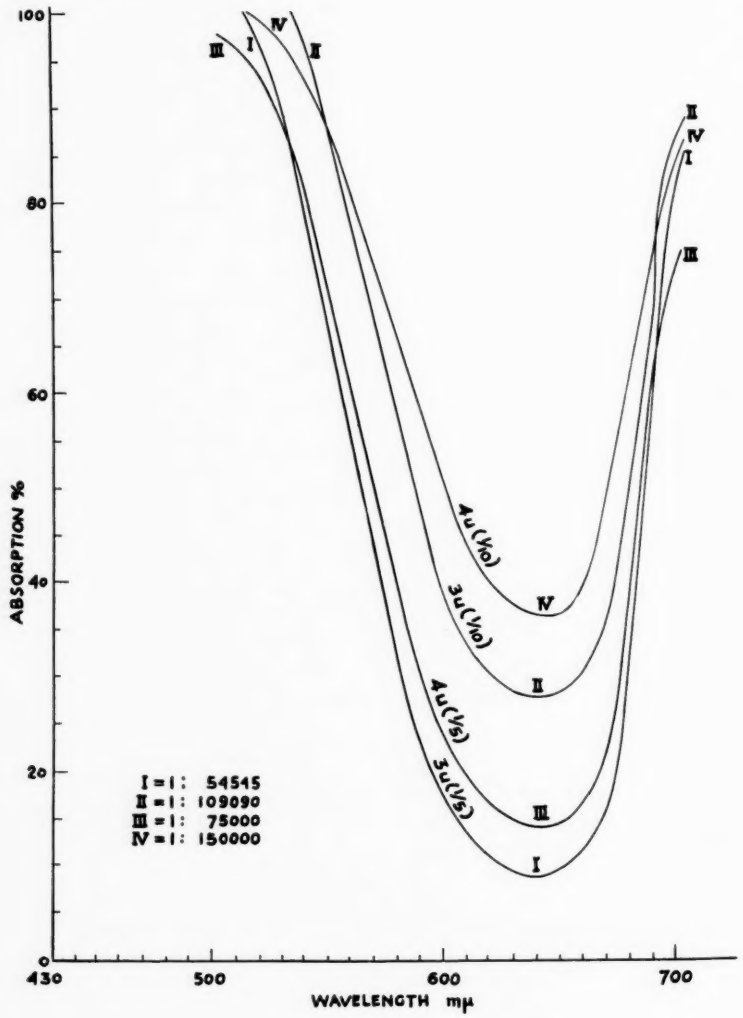

Fig. 1.

In Table I various lots of heparin from different sources were run at the indicated dilutions. These dilutions were calculated to make a solution containing about 1 u per cubic centimeter. The concentrations listed are the averages of three tests each, no concentration varying more than 50 gamma from the average. To arrive at the units per cubic centimeter we subtract the residual concentrations of TB in 3 u and 4 u tests from the original concentration of 1,000 gamma, then divide each by the decolorization value of 1 u or 150 gamma, then divide by 3 u and 4 u, respectively, and average the results. For example, taking

sample No. 1: 3 u = 585 gamma, 4 u = 431 gamma; 1,000 gamma – 585 gamma = 415 gamma, 1,000 gamma – 431 gamma = 569 gamma; 415 gamma ÷ 150 = 2.76 u, 569 gamma ÷ 150 = 3.79 u; 2.76 u ÷ 3 = 0.92 u, 3.79 u ÷ 4 = 0.95 u; 0.92 u and 0.95 u averages 0.935 u.

TABLE I

THE COLORIMETRIC UNIT OF HEPARIN WITH THE DUBOSCQ COLORIMETER AS EXPRESSED BY THE DECOLORIZATION OF A TOLUIDINE BLUE SOLUTION OF 1,000 GAMMA PER 6 C.C. FOLLOWING THE ADDITION OF 1 TO 5 ANTICOAGULANT UNITS OF HEPARIN

NUMBER	PRODUCER	DILUTION	CONCENTRATION OF TOLUIDINE BLUE, γ						U/C.C.	ORIGINAL STRENGTH
			1 U	2 U	3 U	4 U	5 U	CONTROL		
1	CLT	1 mg./110 c.c.	856	713	585	431	341	1,000	0.94	103 u/mg.
2	CLT	1 mg./ 15 c.c.	778	589	385	243	0	1,000	1.31	20 u/mg.
3	CLT	1 mg./110 c.c.	848	663	552	443	326	1,000	0.96	106 u/mg.
4	HLR	1 c.c./400 c.c.	718	519	342	211	0	1,000	1.38	552 u/c.c.
5	HLR	1 c.c./400 c.c.	777	571	397	261	0	1,000	1.28	512 u/c.c.
6	LL	1 c.c./1,100 c.c.	774	641	473	410	298	1,000	1.06	1,166 u/c.c.
7	LL	1 mg./100 c.c.	929	659	505	454	315	1,000	0.99	99 u/mg.
8	LL	1 mg./100 c.c.	776	623	534	493	396	1,000	0.93	93 u/mg.

CLT = Connaught Laboratories; HLR = Hoffmann-LaRoche; LL = Lederle Laboratories.

TABLE II

PHOTOELECTRIC (EVELYN) STUDIES WITH 3 AND 4 COLORIMETRIC UNIT TESTS OF TOLUIDINE BLUE (TB) AND AZURE A (AA)

FILTER NUMBER	THREE UNITS			FOUR UNITS		
Mμ	TB	AA	AVERAGE	TB	AA	AVERAGE
490	60.5	67.5	64.0	67.8	71.8	69.8
520	42.3	48.0	45.2	52.0	57.0	54.5
540	21.0	23.0	22.0	32.0	32.8	32.4
635	0.5	1.5	1.0	1.0	3.8	2.4
660	8.0	15.0	11.5	9.0	18.0	13.5

Center setting = 75. Each value represents average of duplicate tests.

TABLE III

COMPARISON BETWEEN VARIOUS DILUTIONS OF AZURE A OR TOLUIDINE BLUE ON THE EVELYN PHOTOELECTRIC COLORIMETER

FILTER NUMBER	DILUTIONS OF DYE					CENTER SETTING
Mμ	1:6,000	1:12,000	1:15,000	1:18,000	1:30,000	
520	24.0	47.0	55.0	60.5	78.0	75.0
540	7.0	24.5	32.0	38.0	61.0	75.0

From Table II we see that a 4 u test (54.5) equals 1:15,000 dilution (55.0) on Table III at 520 mμ. A 4 u test contains six times 1:15,000 Gm. dye as 100 per cent toluidine blue, since a 4 u test contains 6 c.c. total solution. The decolorization due to 4 u of heparin equals 6 (1:6,000) – 6 (1:15,000) = 36:60,000 = 600:1,000,000 = 600 gamma. Therefore, 1 u heparin decolorizes 150 gamma of 100 per cent toluidine blue.

METHOD II

We have found that in method I there is a possible experimental error of ±50 gamma between triplicate tests. Assuming an error of 40 gamma in testing a solution diluted to 1 c.c. = 1 u, a 3 unit test would leave 590 or 510 gamma, and

a 4 unit test would leave 440 or 360 gamma of dye in solution. If a 3 unit test leaves 590 gamma and a 4 unit test leaves 440 gamma, the value of the solution tested would equal 0.92 u per cubic centimeter. If a 3 unit test leaves 510 gamma and a 4 unit test leaves 360 gamma, the value of the solution tested would equal 1.08 u per cubic centimeter. Referred back to a sample of 100 u per milligram, this would mean an error of ±8 u per milligram. In order to test weighed dry samples in concentrated solution, we developed the following procedure:

Procedure.—A measured portion of the sample is titrated in a beaker with a 1:2,000 solution of TB or AA made up by adding 0.8475 Gm. TB (NU-3) to 1,000 c.c. or 0.5747 Gm. AA (NAz-7) to 1,000 c.c. distilled water. To this solution add 0.05 c.c. increments, with swirling, from a 25 c.c. burette until precipitation starts, allow it to aggregate, and continue to add slowly, drop by drop,

TABLE IV

SAMPLE TITRATIONS WITH 0.05 PER CENT SOLUTIONS OF TOLUIDINE BLUE AND AZURE A

HEPARIN		TITRATIONS IN C.C.				COLORIMETRIC UNITS
IDENTIFICATION	MG.	1	2	3	AVERAGE	PER MG.
Na-50-110	0.454	13.1	13.15	13.1	13.1	96.2
Na-61-110	0.454	12.1	12.2	12.2	12.2	89.7
Na-60-110	0.454	12.0	11.8	11.9	11.9	87.3
Ba-61-100	0.500	13.1	13.1	13.3	13.2	88.0
Ba-62-100	0.500	11.5	11.6	11.5	11.5	76.7
Ba-43-4-100	0.500	12.2	12.25	12.15	12.2	81.3

3 ⅓ u = 1 c.c. of 1:2,000 TB; Na indicates sodium salt; Ba indicates barium salt.

TABLE V

COMPARISON BETWEEN ANTICOAGULANT AND COLORIMETRIC UNITS OF HEPARIN FROM VARIOUS SOURCES

HEPARIN IDENTIFICATION	ANTICOAGULANT UNITS PER MG.	COLORIMETRIC UNITS PER MG.
Lederle Laboratories		
Na-43-4	110	109
Na-45	110	102
Na-50	110	96
Na-61	110	90
Na-60	110	87
Ba-61	100	88
Ba-43-4	100	81
Ba-44	100	79
Ba-62	100	77
Ba63	100	76
Connaught Laboratories		
I	120	108
II	120	98
III	115	93
IV	110	108
V	100	76
Abbott Laboratories		
Na-4	145	106
Ba-1-PHi	95	90
Ba-3-PHi-II	90	90
Ba-2-PHi*	50	77
British Drug House†	85	95
	85	84
Løvens, Copenhagen† Na	85	84

*Dried at 80° C. and 40 mm. vacuum.
†Assayed and kindly supplied by Dr. W. C. Risser.

until a distinct blue solution equal to about 1:40,000 TB is observed among the heparin dye flocculation. The titration in cubic centimeters is multiplied by 3⅓ to obtain unitage in colorimetric units. Titration is materially assisted by bottom lighting under the beaker and it is extremely important that the precipitate be allowed to form slowly in order to avoid overtitration. The amount of sample plus titrating solution should not be greater than 75 c.c. at the end point, for, in the course of our work we find that certain concentrations are necessary to produce precipitation of the dye heparin complex. Considerable dilution before adding TB or the addition of TB of too great dilution will prevent precipitation. Various shades of blue mixed with violet will be the result no matter how great the excess of TB or AA over heparin. In titration at considerable dilution the deep violet color of the TB heparin or AA heparin complex masks the end point and makes a sharp end point impossible, except, possibly, a special filter in a photoelectric colorimeter might determine the end point accurately. However, since it is simpler to precipitate the interfering violet at the end point, we choose to do so. We find that a concentration of approximately 1:40,000 of TB or AA is necessary for complete precipitation.

Results.—From tests conducted on samples, we have found that a 100 unit sample of the purest heparin with regard to our reaction yielded a 30 c.c. titration with 1:2,000 TB. Therefore, we arbitrarily set 1 c.c. of 1:2,000 TB equal to 3⅓ u. One cubic centimeter of 1:2,000 TB equals 0.5 mg. TB; therefore, 1 u equals 0.15 mg. (or 150 gamma).

From triplicate titrations of heparin in Table IV it can be seen that a sharp end point is obtained and that the test is reproducible with rather high accuracy (±2 per cent), and over-all accuracy about ±5 per cent.*

In Table V the anticoagulant units and colorimetric units of heparin from various sources are compared.

DISCUSSION

Relationship Between Azure A and Toluidine Blue.—Our results indicate with regard to heparin that a solution of azure A of the same reactive strength as a solution of toluidine blue shows equal chromatic densities at various dilutions. We have found that a solution of azure A that will give, for example, a 30 c.c. titration with a certain sample of heparin when compared with a solution of toluidine blue that gives a 30 c.c. titration with the same sample of heparin will show the same chromatic concentration. Dr. Conn[2a] stated that toluidine blue has not been satisfactorily standardized, and he refers to the formula[11] published demonstrating that its composition is very similar to azure A. In view of the results we obtained, the question arises as to whether the difference between the two dyes is even as much as has been indicated by the published formulas. In this connection it was found that upon standing a shift in the absorption band occurs.[2a] From this Conn concluded that it would appear that toluidine blue can spontaneously change into azure A. However, such a particular chemical change seems to him to be improbable. He feels that there is another, yet undiscovered, explanation for this phenomenon. Old samples of toluidine blue in which this

*All weighings must be to the third significant figure to obtain ± 2 per cent accuracy.

change in color has occurred yielded the same results as azure A. These findings seem to indicate that it might be difficult to standardize any technique that calls for toluidine blue. However, the present toluidine blue (NU-3) of the National Aniline Chemical Company, which has been made in a way different from earlier lots, has to date not shown this change in color absorption. Our sample (NU-3), which was rechecked by Dr. Conn, did not show any change whatsoever in its absorption maximum and agrees entirely in its optical characteristics with the one he has on hand.*

The sample of NU-3 (dye strength 59 per cent) was compared to azure A (NAz-7) and the NAz-7 showed a dye strength of 87 per cent toluidine blue. It would appear that the NAz-7 has, for instance, 13 per cent impurities as compared to 41 per cent in the NU-3. We wonder if an appreciable amount of leuco products is present in the azure A as seems to be in toluidine blue according to information from the manufacturer.[2a] However, we do not know if leuco compounds have any effect upon the thiazins as to causing change in the chromophore group. Both azure A and toluidine blue gave reproducible results with heparin, and thus we feel that these dyes, as standardized by the Commission on Biological Stains, can be used interchangeably.

Comparison of Anticoagulant and Colorimetric Units of Heparin.—By our test the various samples contributed by different laboratories show variation up to 33 per cent of the label value. This suggests that either our test does not measure the anticoagulant value of the sample as a whole, or else the previous tests used were not based on as reproducible conditions as ours. It may be noted that various biological assay methods were used to determine the unit of heparin.†‡ However, the possibility is present that the heparin samples as prepared at the present time do not owe all their anticoagulant power to the heparin compound alone, but may be modified by plasma factors not identified[13] and possibly present in the albumin fraction.[14, 15] An alternative possibility is that there are present in the samples impurities which modify the toluidine blue or azure A reaction. Nevertheless, we feel, since other polysulfuric acid esters of polysaccharides with comparatively high molecular weight exhibit anticoagulant activity[16, 17] and also metachromatic change, as for instance in chondroitin sulfuric acid,[1, 18] they seem to be of similar chemical composition. Our data may not truly represent the physiological activity of the heparin samples, although the results are proportional to the anticoagulant activity as found by various workers. In this connection, a portion of a sample containing about 95 u per milligram was dried to constant weight, heating at 80° C., under low vacuum of 40 mm. and with a slow stream of dry air drawn over the powder. The anticoagulant activity of this portion was found to be 50 u. By comparing the colorimetric titration we found 90 u in the first portion, 77 u in the second. Although the discrepancy between both portions is about 3½ times larger with the biological assay method, the decrease of 13 units with our method is significant. In a test based on physiological standards, such as cat or ox blood, the

*Personal communication, January 16, 1942.

†Dr. Charles used a modification of Howell's method by Charles and Scott.[12] Dr. Meadows used a modification of the method of Reinert and Winterstein.[9] Dr. Risser used a modification of the method of Charles and Scott.[12]

‡Assays employed by Charles, Risser, and Meadows show accuracies as high as ± 5 per cent.

reliability of such a standard is always open to question. According to Jorpes[19] differences of 15 to 20 per cent were demonstrated with his method of standardization with ox blood. There are seasonal changes reported by him. For instance, he found the tendency to coagulation in the summer was at least twice as great as during the winter. Other factors are mentioned by Jorpes which may affect the coagulation time, such as temperature, species, and admixtures of thromboplastin (thrombokinase). Therefore, we feel justified, in view of the indefinite status of the unit of heparin, in establishing a unit on the basis of these reliably reproducible azure A-toluidine blue reactions with heparin.

Colorimetric Unit of Heparin.—We have demonstrated by Tables II and III that a 4 u test of a sample of the Connaught Laboratories containing 110 u per milligram, which exhibited the strongest colorimetric reaction, decolorized 150 gamma per Murray-Best unit. For convenience in computation we have chosen this sample to define the unit of heparin. Therefore, we present the colorimetric unit of heparin equaling the decolorization of 150 gamma of 100 per cent dye content of toluidine blue. It follows that this unit would equal 254 gamma of toluidine blue NU-3 or 172 gamma of azure A NAz-7. We discover in our titration method (method II) that, using 1 u equaling 150 gamma 100 per cent toluidine blue, our results correspond with results obtained by our decolorization method (method I).

SUMMARY

1. The toluidine blue reaction for heparin was developed by use of the Dubosecq and Evelyn colorimeters and shown to be practical for assay purposes. The same reaction for heparin was found to be specific with azure A.

2. The relationship between azure A and toluidine blue is discussed. With heparin, azure A yielded 87 per cent dye content as toluidine blue compared to 59 per cent dye content toluidine blue.

3. A titration method of positive value for heparin assay was established which yielded rapid results, reproducible to ±2 per cent, over-all accuracy about ±5 per cent.

4. The comparison between anticoagulant and colorimetric units is discussed.

5. The colorimetric unit of heparin is defined. One unit of heparin decolorizes 150 gamma of 100 per cent toluidine blue dye content. This unit equals 254 gamma of toluidine blue NU-3 or 172 gamma of azure A NAz-7.

We wish to thank Dr. A. F. Charles of Toronto, Dr. C. M. Meadows of Pearl River, N. Y., and Dr. W. C. Risser of North Chicago for supplying us with their preparations of heparin. We also wish to thank Dr. H. J. Conn for his courteous cooperation.

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THE QUANTITATIVE ESTIMATION OF GLUCURONATES IN URINE*

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THE need has long been felt for a reliable quantitative method of estimating glucuronates in urine. Such a method would be of special value in determining the extent of exposure of workmen to any compound which had been found to be detoxified by conjugation with glucuronic acid.

Some of the methods reported in the literature require from eighteen to twenty-four hours for a single analysis (Sauer,[1] Kakinuma[2]); others, based on the formation and distillation of furfural, are disturbed if glucides are present, and are not sufficiently precise in dealing with amounts less than 2 mg. (Tanabe,[3] Fürth and Peschke[4]). Venning[5] employed a gravimetric procedure but obtained only approximately correct results. Florkin,[6] and Maughan, Evelyn, and Browne[7] have developed a quantitative method from Tollens'[8] qualitative naphthoresorcinol test, but single estimations were found unreliable. Recently, Meyer, Block, and Chaffee[9] have reported a method for the estimation of uronic acids in pure solutions.

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Principle of the Method.—The method employed here is also a modification of Tollens' original test, but, in contrast to similar procedures previously reported, it makes use of a preliminary hydrolysis at 75° C., a great excess of naphthoresorcinol, and a hydrochloric acid-naphthoresorcinol mixture heated to 50° C. Further, the final estimation is carried out with a photoelectric spectrometer or with a photoelectric colorimeter.

Experiments have shown that few compounds will interfere in this determination and that an occasional urine sample requires preliminary treatment. If urine contains no pentose or glucose, then the regular procedure is followed. However, if the concentration of pentose is found to be about 0.03 per cent or that of glucose is about 0.3 per cent, the sample analyzed should then be reduced to about 0.1 ml., and when the concentration of these sugars exceeds these limits, the urine samples should then be freed of them prior to hydrolysis. The presence of 1.0 per cent protein or 0.2 per cent ascorbic acid does not interfere. (The normal excretion of ascorbic acid ranges from 30 to 50 mg. in twenty-four hours,[11] which is not in excess of 0.025 mg. in 0.5 ml. of urine. It was found that when 0.5 mg. of ascorbic acid and 0.12 mg. of glucuronic acid were added to a 0.5 ml. sample of human urine, the recoveries of glucuronic acid ranged from 0.124 to 0.132 mg.)

Reagents Required.—Hydrochloric acid (19 Gm. in 100 ml.).

Naphthoresorcinol* (10 Gm. in 100 ml. of 95 per cent ethyl alcohol). This reagent is centrifuged or filtered and will remain stable for five days if kept in a cool place protected from light.

Ether, C.P.

METHOD OF ANALYSIS

(1) *Determining the Need for Removing Interfering Substances.*—The presence of glucose is readily determined by Benedict's qualitative test. Amounts of ascorbic acid sufficient to cause interference may be suspected only in cases of vitamin medication. Testing for pentoses may be carried out by Tollens' test slightly modified as follows: To 1 ml. of urine and 1 ml. of concentrated hydrochloric acid is added 0.1 ml. of 10 per cent naphthoresorcinol in 95 per cent ethyl alcohol. The mixture is boiled for one minute in a test tube with constant shaking, permitted to stand at room temperature for four minutes, cooled under the tap, and extracted with 10 ml. of ether. When the urine is free of pentose, the ether is purple (with the exception of unusual cases that contain no glucuronic acid at all); in the presence of 0.1 per cent pentose the ether is colorless, while intermediate concentrations give various shades of purple. (This unusual behavior seems to result from the speed of reaction of pentose with naphthoresorcinol. The latter appears to be faster than the speed with which glucuronic acid and naphthoresorcinol react. Hence if pentose and glucuronic acid are both present in a sample of urine, pentose will react first, forming a brownish-black ether-insoluble pigment; the absence of color in the ether layer is then due to the fact that the naphthoresorcinol has been used up. The preliminary hydrolysis in the quantitative procedure makes a large amount of "free" glucuronic acid available for immediate reaction. The high concentra-

*Purchased from B. L. Lemke, 248 West Broadway, New York City.

tion of naphthoresorcinol is needed, because this compound reacts not only with glucuronic acid but probably with many impurities; an excess furnishes enough for reaction with the urinary constituents that form ether-insoluble substances and for the reaction with glucuronic acid whereby the purple ether-soluble color is produced. These two factors are mainly responsible for the fact that glucuronic acid can be satisfactorily determined in relatively large urine samples [0.5 ml.] without removal of interfering substances. To study the color, it is best to set up a few specimens of normal urine to which have been added known amounts of arabinose.)

(2) *Removal of Interfering Substances.*—The removal of interfering substances is accomplished by precipitation with lead acetate in slightly acid solution, as suggested originally by Quick,[10] and the precipitation of conjugated glucuronates with basic lead acetate, according to Tollens.[8]

A small quantity (0.2 to 0.4 ml.) of the urine to be tested is measured into a 15 ml. conical centrifuge tube, diluted to 2 ml. with water, and made acid to litmus with 1 to 3 drops of glacial acetic acid. Then, 0.2 ml. of 5 per cent lead acetate is added and the sample is mixed and centrifuged. Tests should be made for complete precipitation of interfering substances by the addition of a drop of lead acetate. If precipitation is found to be incomplete, an additional 0.1 ml. of lead acetate solution is added, the mixture is shaken and again centrifuged. This procedure is repeated until precipitation is complete. The supernatant fluid is poured into a similar centrifuge tube and 0.3 ml. of 10 per cent sodium hydroxide is added. If a precipitate does not form, more sodium hydroxide is added until a permanent precipitate is obtained. Then 3 ml. of the basic lead acetate solution* are added, and the contents are mixed and centrifuged. Tests should be made for complete precipitation of glucuronates by the further addition of a drop of basic lead acetate. When precipitation is complete, the supernatant fluid is discarded and the precipitate is washed by thoroughly stirring it with 10 ml. of water. The mixture is centrifuged again, the supernatant liquid is discarded. The residue is transferred quantitatively, employing 5 ml. of concentrated hydrochloric acid followed by 5 ml. of water, into a 50 ml. graduated cylinder and then incubated for fifteen minutes at 75° C. One milliliter of 10 per cent naphthoresorcinol is added, and the cylinder is allowed to stand for ninety minutes in a water bath maintained at 50° C. After cooling under the tap, the solution is extracted with ether and the remainder of the determination is carried out as described below.

(3) *Analysis.*—(The analysis is started at this point if interfering amounts of glucose, pentose, protein, or ascorbic acid are known to be absent.)

The sample of urine is mixed well, and its total volume is determined. In the case of human or dog urine, 0.3 to 0.5 ml., and of rabbit's urine, 0.2 to 0.4 ml., is then pipetted into a 50 ml. glass-stoppered graduated cylinder and diluted with water to 5 ml. After the addition of 0.2 ml. of 19 per cent hydrochloric acid, the whole is kept for forty-five minutes in a water bath at 75° C.; 5 ml. of concentrated hydrochloric acid and 1 ml. of 10 per cent naphthoresorcinol are then added, and the cylinder is allowed to stand for ninety minutes in a water

*Prepared by heating 10 Gm. of dibasic lead acetate with 200 ml. of water; after boiling and stirring for a few minutes, the solution is allowed to cool and is filtered.

bath maintained at 50° C. After cooling under the tap, the solution is extracted with 10 ml. of ether by shaking the cylinder vigorously for five seconds. After separation into two layers, sufficient ether is added to bring the ether volume to exactly 15 ml., and it is mixed by gently rotating the cylinder. Addition of the second portion of ether will completely clear up any slight turbidity caused by water in the upper layer.

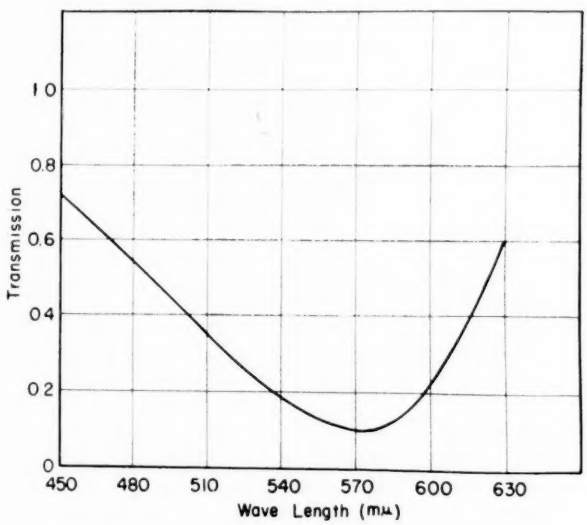

Fig. 1.—Transmission curve of the ether extract.

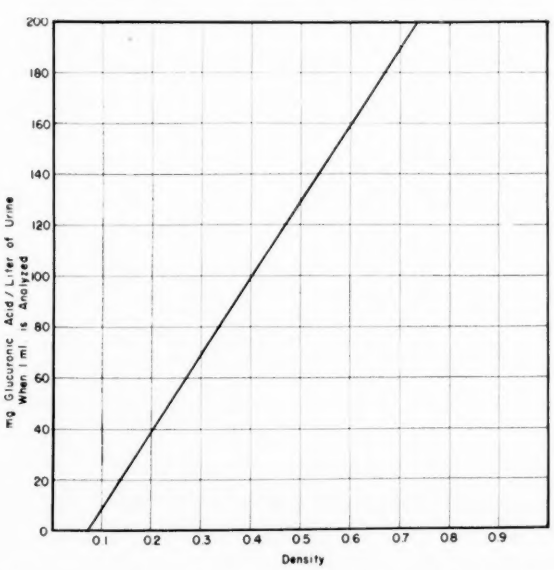

Fig. 2.—Glucuronic acid. Concentration versus density measured with the spectrophotometer at 570 mμ employing 10 mm. matched cells.

(4) *Colorimetric Reading.*—The colored ether is now pipetted into a glass-stoppered flask and is ready for spectrophotometric analysis, which is carried out by using 10 mm. matched cells, with the instrument set at the wave length

of 570 mμ. The final estimation may also be carried out, with equally good results, with a Leitz photoelectric colorimeter employing the green filter No. F-401.

Fig. 1 gives the transmission curve of the pigment formed under the conditions outlined in this procedure.

Fig. 2 gives the relationship between density and concentration of glucuronic acid per liter of urine when the photoelectric spectrometer is employed.

Fig. 3 gives the relationship between concentration of glucuronic acid per liter of urine and readings with the photoelectric colorimeter.

Figs. 2 and 3 were prepared by plotting the readings obtained when known aqueous solutions of pure glucuronic acid (or of a conjugated glucuronate) were analyzed by this procedure.[12]

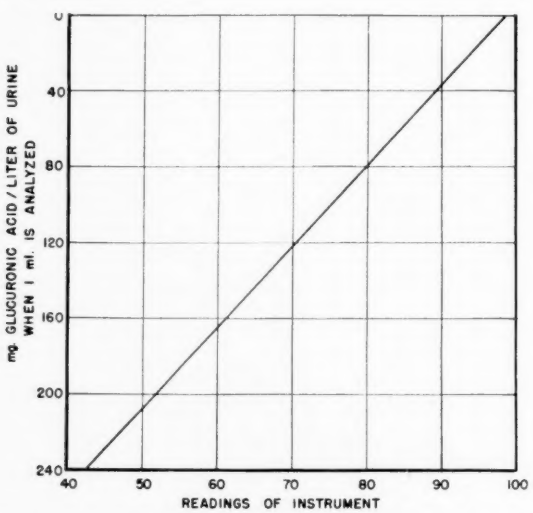

Fig. 3.—Glucuronic acid. Concentration versus reading with the Leitz photoelectric colorimeter with green filter No. F-401.

(5) *Calculation.*—The following example will serve to illustrate the calculation: if 0.25 ml. from a total volume of 280 ml. is analyzed, and a concentration of 70 obtained from Fig. 2 or Fig. 3, then:

$$\frac{70}{1{,}000}\ \text{mg.} \times \frac{1}{0.25} \times 280 \rightarrow 78.4\ \text{mg. glucuronic acid/total volume.}$$

(6) *Certain Practical Considerations.*—

(a) The alcoholic naphthoresorcinol solution employed must be *perfectly clear.* The amounts of impurities in this compound vary; it is, therefore, advisable to plot new curves with each new lot of naphthoresorcinol.

(b) The colored ether solution is stable for about one hour; it should be protected from direct sunlight. (Prolonged standing causes an increase in the intensity of the color.)

(c) The analysis must be carried out without delay after the addition of hydrochloric acid. If it should be found necessary to remove interfering substances from the urine, a convenient point to interrupt the analysis comes after the precipitation of glucuronates with basic lead acetate.

(d) An excess of acid lead acetate must be avoided because this will result in loss of glucuronates.

(e) Normal human urine suffers no loss of glucuronic acid when permitted to stand for a week at room temperature without preservatives.

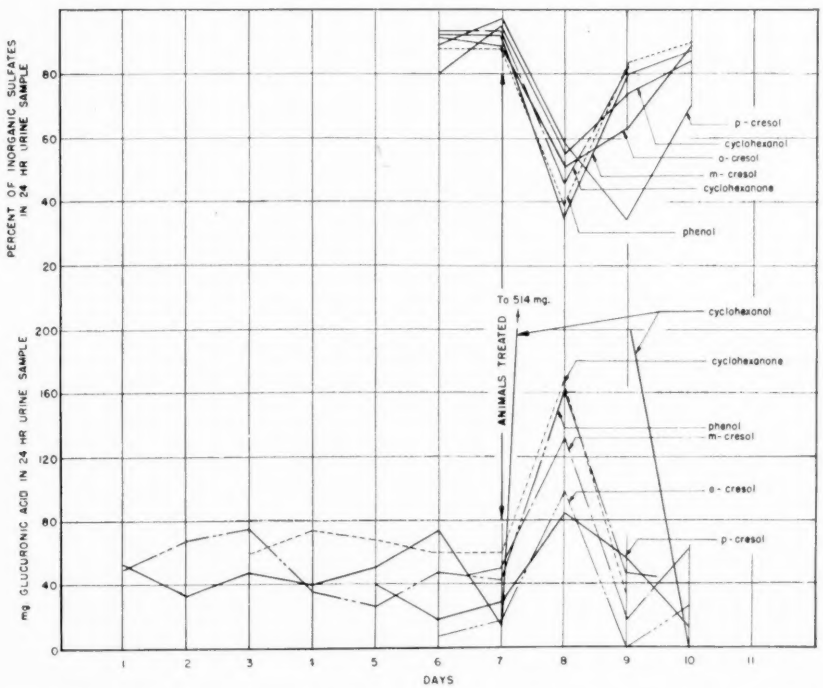

Fig. 4.—Excretion of inorganic sulfates and glucuronic acid in the urine of rabbits following a single oral administration of about half the lethal dose of phenol, o-, m-, and p-cresol, cyclohexanol, and cyclohexanone, respectively.

EXPERIMENTAL

Table I summarizes the recoveries of free and conjugated glucuronic acid added to normal human or rabbit urine and analyzed without previous removal of interfering substances. The recoveries are satisfactory. Table II gives the amounts of free and conjugated glucuronic acid recovered after their addition to urine containing glucose or arabinose; here again the urine was not subjected to preliminary treatment. It can be seen that the presence of 0.03 per cent arabinose or 0.5 per cent glucose does not interfere with recovery of the conjugated compound; the recovery of free glucuronic acid is slightly less complete. Table III shows that following the removal of interfering substances from the urine, as outlined above, 0.25 per cent arabinose added to the urine is removed to a sufficient degree to enable recovery of the conjugated glucuronates; a slight loss of glucuronates is noted, however, when urine contains 0.5 per cent of this pentose. Table IV summarizes the excretion of glucuronic acid in twenty-four-hour specimens from normal men who were under no dietary restrictions. The daily excretions ranged from 65 to 239 mg. These amounts bore no relationship to the total volume of urine excreted. A young dog on a diet consisting

TABLE I

RECOVERY OF FREE AND CONJUGATED GLUCURONIC ACIDS ADDED TO NORMAL URINE*

SAMPLE	COMPOUND	
	ADDED (MG.)	RECOVERED (MG.)
	Glucuronic acid	
0.5 ml. human urine	0.06	0.07
0.5 ml. human urine	0.12	0.13
0.5 ml. human urine	0.18	0.19
0.25 ml. rabbit urine	0.12	0.10
0.25 ml. rabbit urine	0.18	0.17
0.4 ml. rabbit urine	0.12	0.15
	Borneolglucuronic acid	
0.5 ml. human urine	0.06	0.07
0.5 ml. human urine	0.06	0.05
0.5 ml. human urine	0.12	0.14
0.5 ml. human urine	0.18	0.19
0.5 ml. human urine	0.18	0.17
0.1 ml. rabbit urine	0.12	0.12
0.1 ml. rabbit urine	0.24	0.21
	Mentholglucuronic acid	
0.25 ml. human urine	0.06	0.07
0.25 ml. human urine	0.12	0.14
0.25 ml. human urine	0.24	0.24
0.25 ml. human urine	0.24	0.20
0.4 ml. rabbit urine	0.13	0.10
0.1 ml. rabbit urine	0.06	0.08
0.1 ml. rabbit urine	0.18	0.18
0.1 ml. rabbit urine	0.24	0.21

*Interfering substances were not removed.

TABLE II

RECOVERY OF GLUCURONIC ACID ADDED TO URINE CONTAINING GLUCOSE OR ARABINOSE*

SAMPLE 0.1 ml. OF RABBIT URINE CONTAINING	COMPOUND	
	ADDED (MG.)	RECOVERED (MG.)
	GLUCURONIC ACID	
0.3% glucose	0.06	0.05
0.3% glucose	0.12	0.11
0.3% glucose	0.18	0.19
0.5% glucose	0.03	0.02
0.5% glucose	0.06	0.05
0.5% glucose	0.12	0.09
0.03% arabinose	0.03	0.02
0.03% arabinose	0.06	0.04
0.03% arabinose	0.12	0.10
0.1% arabinose	0.18	0.14
0.1% arabinose	0.13	0.09
0.5% arabinose	0.12	0
0.5% arabinose	0.12	0.04
0.5% arabinose	0.12	0.03
	BORNEOLGLUCURONIC ACID	
0.5% glucose	0.06	0.06
0.5% glucose	0.12	0.14
0.5% glucose	0.18	0.19
0.5% glucose	0.18	0.17
0.5% glucose	0.18	0.16
0.5% glucose	0.18	0.19
0.5% glucose	0.18	0.17
0.03% arabinose	0.12	0.11
0.03% arabinose	0.18	0.17
0.05% arabinose	0.12	0.09
0.05% arabinose	0.18	0.15
0.1% arabinose	0.12	0.08
0.1% arabinose	0.18	0.13
0.5% arabinose	0.18	0

*Interfering substances were not removed.

TABLE III

RECOVERY OF GLUCURONIC ACID ADDED TO URINE AFTER REMOVAL OF ARABINOSE AND GLUCOSE

SAMPLE 0.1 ml. OF RABBIT URINE CONTAINING	COMPOUND	
	ADDED (MG.)	RECOVERED (MG.)
	GLUCURONIC ACID	
0.1% arabinose	0.13	0.11
0.25% arabinose	0.12	0.08
0.25% arabinose	0.12	0.09
0.5% arabinose	0.12	0.09
0.5% arabinose	0.12	0.07
0.5% glucose	0.12	0.11
	BORNEOLGLUCURONIC ACID	
0.25% arabinose	0.06	0.05
0.25% arabinose	0.06	0.04
0.25% arabinose	0.12	0.13
0.25% arabinose	0.12	0.13
0.25% arabinose	0.12	0.10
0.25% arabinose	0.12	0.10
0.5% arabinose	0.06	0.06
0.5% arabinose	0.06	0.04
0.5% arabinose	0.12	0.08
0.5% arabinose	0.12	0.08
0.5% arabinose	0.12	0.10

TABLE IV

GLUCURONIC ACID CONTENT OF NORMAL HUMAN URINE*

(The volume of samples analyzed ranged from 0.25 to 0.5 ml.)

CASE	VOLUME OF THE 24-HOUR SPECIMEN (ML.)	GLUCURONIC ACID IN THE 24-HOUR URINE SPECIMEN (MG.)
LD	370	117
LD	465	114
FW	500	125
RH	540	139
HP	600	65
WD	660	128
HD	750	83
HP	760	84
RH	775	101
WD	795	139
WD	795	186
LD	800	105
RH	845	130
WH	870	158
WD	885	224
FW	925	159
HD	945	120
WH	950	163
WD	950	104
WD	1,003	123
HP	1,070	75
AB	1,100	239
HD	1,100	154
FF	1,260	86
GN	1,350	142
GN	1,565	139
WD	1,640	164
GN	1,945	154
GN	2,025	146

*The urine was not subjected to preliminary treatment because specimens did not contain glucose, pentose, or protein in interfering quantities and the subjects had not received ascorbic acid as medication.

of beef heart and "kibbles" (dried dog food) excreted daily for six days from 24 to 108 mg. of glucuronic acid, and rabbits on a diet of Purina pellets excreted daily for eighteen days from 15 to 75 mg.

Fig. 4 shows the drop in inorganic sulfates and the corresponding increased output of glucuronic acid in rabbits following administration of half the lethal oral dose of phenol, o-, m-, and p-cresol, cyclohexanone, and cyclohexanol, respectively.

SUMMARY

1. A quantitative procedure is presented for the estimation of glucuronates in urine. The method is sufficiently reliable for clinical and industrial use.

2. The daily output of glucuronates for men on an unrestricted diet ranged from 65 to 239 mg. per twenty-four hours. A dog excreted from 24 to 108 mg. and rabbits from 15 to 75 mg. for one-day periods.

3. The administration of about half the lethal oral dose of phenol, o-, m-, and p-cresol, cyclohexanol, and cyclohexanone, respectively, produces, in addition to the well-known drop in the output of inorganic sulfates, a sharp rise in the excretion of glucuronates.

The borneolglucuronic and the mentholglucuronic acids were prepared and kindly supplied by Dr. Armand T. Quick of Marquette University School of Medicine; the glucuronic acid was kindly supplied for these studies by J. R. Harrower of the University of Wisconsin.

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THE DETERMINATION OF BROMIDES AND CHLORIDES IN BIOLOGICAL MATERIALS AND AN ACCURATE CLINICAL METHOD FOR DETERMINING BROMIDE IN SMALL AMOUNTS OF BLOOD*

LEON A. GREENBERG, NEW HAVEN, CONN.

IN CARRYING out a series of investigations on the effects of ingested bromides, the needs arose for: (1) a rapid method for determining accurately both bromides and chlorides in a single wet sample of tissue or blood; and (2) a rapid and reliable clinical method for determining the concentration of bromide in 0.1 c.c. of blood as drawn from a needle prick. Trial of the various methods for determining bromides and chlorides reported in the literature showed that none fully satisfied these needs. Two procedures which did satisfy them were, therefore, developed and are reported here. The findings from analyses made on predetermined amounts of bromide and chloride are given to show the accuracy of the methods and in addition the results from a series of determinations on the comparative bromide content of whole blood and serum.

All determinations of bromide in biological material are carried out in two stages: (1) the treatment of the material to obtain the bromide in a form and medium suitable for estimation; and (2) the estimation of the bromide. Three general methods are available for carrying out the first stage: (1) deproteinization; (2) destruction of the dried organic material by alkaline ashing; and (3) destruction by acid digestion.

TREATMENT OF MATERIAL

Deproteinization can be applied only to fluids; it imposes limitations also on the methods for estimating the bromide. It has been used for the electrometric titration and colorimetric estimation of bromide in blood, serum, and cerebrospinal fluid, but not previously for direct chemical estimation, as in the clinical method to be described later.

Alkaline ashing is the treatment most commonly used. A review of the literature shows, however, that it has given unsatisfactory results in the hands of many investigators. The method in general requires the use of dried material and is open to errors from spattering, overheating, and transfer of materials. Neufeld[1] has described a modification of the Francis and Harvey method with which he obtained accurate recoveries, but the procedure required six to eight hours for a single determination. Brodie and Friedman[2] have used a type of ashing described by Kendall[3] and report that a complete determination may be made in about two hours with accurate recoveries. In this laboratory we have not been able to obtain consistently accurate results with alkaline ashing procedures.

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Acid digestion, except in the form of the closed Carius[4] procedure, has been used much less commonly than the alkaline ashing. It has the advantage that it is a wet digestion and it is rapid. It has, however, the particular disadvantage that the bromide must first be converted to an insoluble salt to prevent loss and then after the digestion reconverted to soluble bromide for estimation. Hartner,[5] failing to obtain satisfactory results with alkaline ashing, used concentrated nitric acid in the presence of silver nitrate. He converted the silver bromide to soluble bromide by treatment with zinc and sodium sulfide. Doering,[6] using the closed Carius method, found that this conversion was not always quantitative and employed zinc only. The procedure took many hours and any advantage of speed of the acid digestion over the alkaline ashing was lost. The procedure to be described in the next section as a laboratory method for the determination of bromide and chloride in tissue employs acid digestion but successfully overcomes the slow conversion of the silver halides with zinc by dissolving them in sodium thiosulfate solution and recovering the soluble bromide.

ESTIMATION OF BROMIDE

Four methods, each with various advantages and disadvantages, are commonly used for estimating bromides after treatment of the material: (1) colorimetric determination; (2) electrometric titration; (3) the liberation and estimation of bromide; and (4) the oxidation of the bromide to bromate and determination by iodometric titration.

For colorimetric determination of bromide the reagents used have been fluorescein,[7] fuchsin,[8] and gold chloride.[9] All colorimetric methods have proved poor because of their lack of sensitivity, inaccuracy, and nonspecificity. A method using gold chloride first described by Walter[9] and later modified by Wuth[10] and by Diethelm[11] has, in spite of its inaccuracy and unreliability, been used in many clinical investigations because of its simplicity.

Electrometric titration of bromide requires either that the bromide be present in concentrations approximating that of chloride present or the preliminary removal of most of the chloride. Its usefulness is correspondingly limited.

Several methods have been described for the oxidation of bromide to free bromine.[12-14] All of them necessitate an exact regulation of conditions to avoid the simultaneous formation of chlorine, incomplete oxidation of bromides, or loss of bromine.[1] All the procedures are time-consuming.

The oxidation of bromide to bromate is quantitative in the presence of chloride and the procedure for carrying it out is rapid and simple. The method originating with van der Meulen,[15] and modified by Kolthoff and Yutzy,[16] involves the oxidation of bromide to bromate by the use of hypochlorite according to the reaction:

$$3\ ClO^- + Br^- = BrO_3^- + 3\ Cl^-.$$

After the oxidation is complete, the excess hypochlorite is reduced with sodium formate. The addition of iodide to the bromate in acid solution results in the liberation of six equivalents of iodine, thus giving a sixfold increase in sensitivity over methods in which the bromine is titrated directly. This oxidation procedure has been used successfully by Dixon[17] and by Brodie and Friedman.[2]

LABORATORY METHOD FOR DETERMINING BROMIDE AND CHLORIDE IN BIOLOGICAL MATERIALS

The method described here has proved to be rapid and highly accurate; it avoids many difficulties inherent in methods previously described and permits the determination of both bromide and chloride on a single sample of material. The material is digested with nitric acid in the presence of a known quantity of silver nitrate. The digestion requires about fifteen minutes. The fluid containing the excess of silver nitrate is separated from the precipitate of silver halides and is titrated according to the procedure of Volhardt; from the value obtained the total halogen content of the material is calculated. The precipitate of silver halides is then dissolved in a solution of sodium thiosulfate; the thiosulfate is destroyed and its products are removed by heating with hydrochloric acid in the presence of metallic zinc. Metallic silver is precipitated. The dissolved zinc is precipitated with sodium carbonate and the filtrate contains all the halides in the form of sodium salts. The bromide is then oxidized by the method of Kolthoff and Yutzy[16] and the bromate is determined iodometrically. The detailed steps of the analytical procedure are as follows:

To the weighed sample (about 1 Gm.) of biological material in a 125 c.c. Erlenmeyer flask there are added exactly 3 c.c. of 0.05 N silver nitrate solution in concentrated nitric acid and also 25 c.c. of concentrated nitric acid. The mixture is placed on a hot plate for fifteen minutes for digestion. Saturated potassium permanganate solution is then added, drop by drop, until its color persists for several minutes; a few drops of 20 per cent sodium oxalate solution are added to remove the excess permanganate. The flask is then cooled under water flowing from a tap and placed in a rack so tilted that the precipitate of silver halides settles in a small area. The clear supernatant fluid is decanted into a second Erlenmeyer flask; the precipitate is washed twice with 10 c.c. of water from a washing bottle and the decanted fluid is added to that in the second flask. The silver nitrate in this fluid is titrated with thiocyanate and ferric alum after the method of Volhard.[18] The silver nitrate, as determined, when subtracted from that added, gives the total halide content of the material under analysis.

The bromide in the precipitate is then determined as follows: The precipitate is dissolved in 2 c.c. of 20 per cent sodium thiosulfate solution. Twenty cubic centimeters of distilled water are added and 10 c.c. of N hydrochloric acid and the mixture is boiled for five minutes. The flask is then removed from the hot plate and a pinch of powdered zinc is added; the mixture is then boiled vigorously for three minutes to remove the hydrogen sulfide formed. The flask is then removed from the heat and 5 c.c. of 20 per cent potassium carbonate are added to the solution which is then cooled and washed into a volumetric container to 50 c.c. volume. The solution is then filtered and 40 c.c. of the filtrate are placed in a 125 c.c. Erlenmeyer flask.

By the above procedure the silver bromide is converted to a soluble bromide which is then estimated by conversion to bromate followed by iodometric titration after the general procedure of Kolthoff and Yutzy as follows: 2 N hydrochloric acid is added until the acid is in slight excess. Approximately 2.5 Gm. of sodium dihydrogen phosphate and 6 c.c. of sodium hypochlorite solution (1 N in 0.1 N sodium hydroxide) are added, and the mixture is boiled gently for five min-

utes. Five cubic centimeters of 50 per cent sodium formate solution are added. The mixture is then boiled vigorously for five minutes and cooled, after which 5 Gm. of sodium dihydrogen phosphate, 20 c.c. of 2 N hydrochloric acid, 3 drops of 10 per cent ammonium molybdate solution and 1 Gm. of potassium iodide are added. The liberated iodine is titrated with 0.005 N thiosulfate; the titration is completed in the presence of 5 c.c. of 1 per cent starch solution. A blank determination is run whenever new reagents are made up, and the volume of thiosulfate used in this titration is subtracted from that used in analysis. Each cubic centimeter of thiosulfate is equivalent to 66.6 micrograms of bromine in the material used for analysis.

TABLE I

RECOVERIES OF BROMIDE AND CHLORIDE FROM AQUEOUS SOLUTIONS

BROMIDE ADDED (MG./GM.)	CHLORIDE ADDED (MG./GM.)	BROMIDE FOUND (MG./GM.)	CHLORIDE FOUND (MG./GM.)
0.388	1.52	0.383	1.55
		0.389	1.48
		0.387	1.53
	3.04	0.379	3.02
		0.382	3.04
		0.385	3.00
0.776	1.52	0.770	1.54
		0.778	1.52
		0.775	1.54
	3.04	0.771	3.03
		0.779	3.06
		0.780	3.02
1.552	1.52	1.548	1.51
		1.547	1.51
		1.554	1.55
	3.04	1.555	3.00
		1.554	3.04
		1.552	3.04
2.330	1.52	2.330	1.56
		2.321	1.52
		2.335	1.50
	3.04	2.321	3.03
		2.332	3.02
		2.336	3.05

In Table I are given the results from a series of 24 determinations made on four different aqueous solutions containing known amounts of bromide and chloride. The range of errors for bromide is +0.006 to –0.009 mg. and the algebraic mean is –0.00015; the corresponding values for chloride are +0.04, –0.04, and –0.004.

In Table II are given the results from a series of 12 determinations made on single specimens of blood to which known amounts of chloride and bromide were added. The amount of bromide and chloride in the blood before addition was taken as the average for six determinations. The range of error for bromide is +0.004 to –0.008, and the algebraic mean is –0.0015.

In Table III are given the results of triplicate analyses made on various tissues of an animal previously given sodium bromide. The range of errors in any one series of determinations is 0.005 mg. for bromide and 0.03 mg. for chloride.

TABLE II

RECOVERIES OF BROMIDE AND CHLORIDE FROM BLOOD

(In excess of normal content*)

BROMIDE ADDED (MG./GM.)	CHLORIDE ADDED (MG./GM.)	BROMIDE FOUND (MG./GM.)	CHLORIDE FOUND (MG./GM.)
0.388	1.52	0.389	1.51
0.388	1.52	0.380	1.52
0.388	1.52	0.385	1.55
0.388	1.52	0.388	1.50
0.388	1.52	0.387	1.55
0.388	1.52	0.381	1.54
1.552	1.52	1.548	1.53
1.552	1.52	1.551	1.50
1.552	1.52	1.550	1.52
1.552	1.52	1.555	1.52
1.552	1.52	1.552	1.54
1.552	1.52	1.556	1.50

*Average of six determinations: bromide 0.005 mg. per gram and chloride 5.74 mg. per gram.

TABLE III

RECOVERIES OF BROMIDE FROM TISSUES

TISSUE	BROMIDE PER GRAM WET TISSUE (MG.)	CHLORIDE PER GRAM WET TISSUE (MG.)
Blood	1.742	2.93
	1.747	2.91
	1.743	2.90
Liver	0.722	1.23
	0.719	1.20
	0.720	1.22
Spleen	0.891	1.61
	0.888	1.62
	0.889	1.58
Muscle	0.520	0.88
	0.520	0.90
	0.522	0.88
Heart	0.643	1.11
	0.646	1.09
	0.646	1.10

CLINICAL METHOD FOR DETERMINING BROMIDES

Many procedures described in the literature for the determination of bromide in blood are called micromethods. "Micro" as used in these instances refers to the minute amounts of bromide estimated and not the amount of blood used for the analysis. To the clinician, however, a micromethod is usually one by which clinically important concentrations of the substance being estimated can be determined on a "micro" sample. The methods previously reported for estimating the bromide in blood require 3 to 10 c.c. and hence necessitate venepuncture, and even with these large samples the clinical methods ordinarily employed are so inaccurate as to render them little more than indicators of the general magnitude of the bromide present. The method described here requires only 0.1 c.c. of blood which can readily be obtained by puncture of the

skin of the finger tips or ear lobes. The general procedures consist in deproteinization of the blood, the oxidation of the bromide in the filtrate to bromate, and the iodometric titration of the bromate. The procedure in detail is as follows:

The 0.1 c.c. of blood is discharged directly from the pipette in which it is drawn into 6.5 c.c. of 0.45 per cent zinc sulfate solution in a test tube. One-half cubic centimeter of 0.1 N sodium hydroxide solution is then added and the tube is shaken, placed in a boiling water bath for three minutes, and then cooled. The fluid is next filtered and 5 c.c. of the filtrate are placed into a second test tube. There are then added in succession, 0.3 c.c. of 2 N hydrochloric acid, 1 c.c. of calcium hypochloride solution,* and a pinch of calcium carbonate. The carbonate should be slightly in excess, as indicated by its persistence as a precipitate. The tube is then placed in a boiling water bath for eight minutes, after which 0.4 c.c. of 20 per cent sodium formate solution is added, and the tube is heated for eight minutes more. The tube is then removed from the bath and cooled. There are added in succession 3 c.c. of 2 N hydrochloric acid and 1 c.c. of 10 per cent potassium iodide solution. The tube is shaken and 0.5 c.c. of starch solution is added. The contents are then titrated with 0.0004 N sodium thiosulfate solution. A blank determination is made whenever new reagents are prepared; the blank ordinarily ranges between 1.0 and 1.7 c.c. of thiosulfate and should be subtracted from the volume of thiosulfate used in analysis. Each cubic centimeter of 0.0004 N thiosulfate corresponds to 7.6 mg. per 100 c.c. of bromide or 9.5 mg. per 100 c.c. of sodium bromide in the blood upon which the determination is made.

In Table IV are given the results of a series of 50 determinations made on blood (containing less than 0.05 mg. per 100 c.c. of sodium bromide) to which amounts of sodium bromide, ranging from 50 to 300 mg. per 100 c.c., had been added. The range of absolute errors is fairly constant for all concentrations of bromide and is +6 to –5 mg. per 100 c.c.; the percentage error, therefore, de-

TABLE IV

RECOVERIES OF SODIUM BROMIDE ADDED TO BLOOD

KNOWN CONCENTRATIONS OF SODIUM BROMIDE (MG./100 C.C.)	CONCENTRATION OF SODIUM BROMIDE FOUND (MG./100 C.C.)	RANGE OF ERRORS	
		MG./100 C.C.	PER CENT OF SODIUM BROMIDE PRESENT
25	22, 28, 24, 25, 28, 26, 23, 27, 24, 27	+3, -3	+12, -12
50	47, 52, 46, 48, 53, 50, 55, 46, 46, 52	+5, -4	+10, - 8
100	103, 98, 96, 104, 105, 95, 99, 97, 102, 102	+5, -5	+ 5, - 5
150	153, 147, 146, 150, 150, 156, 149, 151, 153, 149	+6, -4	+ 4, - 3
200	204, 195, 196, 202, 200, 198, 203, 199, 200, 202	+4, -4	+ 2, - 2
300	297, 299, 298, 304, 300, 301, 297, 300, 306, 303	+6, -3	+ 2, - 1

*A 15 per cent solution of $Ca(OCl)_2$ is filtered and the filtrate is diluted with water 1:2.

creased correspondingly with the increase in concentration. At 25 mg. per 100 c.c. in the blood the maximum error was 12 per cent, at 100 mg. per 100 c.c., 5 per cent, and at 300 mg. per 100 c.c., 2 per cent. Such errors are of no clinical significance, particularly as concentrations below 100 mg. per 100 c.c. rarely have clinical importance. In addition to the series of determinations shown in Table IV, analyses were made of the bromide content of the blood of 120 subjects known to have taken no bromide medicinally; the concentrations found ranged from 0 to 5 mg. per 100 c.c., values of no clinical importance. A total of more than 11,000 determinations have been made with the clinical method described and the majority in duplicate with no indications of error beyond the ranges given here.

CONCENTRATION OF BROMIDE IN WHOLE BLOOD AND SERUM

The concentration of bromide is ordinarily recorded clinically as sodium bromide in the serum. The method as described here is applied to whole blood. The analytical method is entirely applicable to serum, but the use of whole blood does away with the necessity of drawing large samples of blood. The concentration of bromide in the serum is somewhat greater than in the whole blood, since, as with chloride, there is less bromide in the corpuscles than in the serum. The difference is, however, slightly less than for chlorides[19]; the relation appears to be approximately 1:0.7. In the clinical determination of bromides even a considerable variation in the number of red blood cells would not introduce a large error. Thus with a bromide distribution of 0.7, 100 mg. per 100 c.c. of bromide in whole blood would at a hematocrit reading of 50 indicate a serum of 118 mg. per 100 c.c.; at 40, 114 mg. per 100 c.c.; at 30, 110 mg. per 100 c.c.; and at 20, 106 mg. per 100 c.c.

A series of determinations was made to relate the concentrations of bromide in serum and whole blood. One hundred and thirty-one analyses were made of blood and serum on 50 different subjects who were receiving, or had recently received, bromide and showed concentrations in the blood ranging between 25 and 190 mg. per 100 c.c. Blood was drawn by venepuncture, oxalate was used as an anticoagulant, and a specimen of the whole blood and of the serum obtained from it were analyzed for bromide. The ratio of bromide in whole blood to that in serum averaged 0.88, with extremes of 0.94 and 0.87. Thus the concentration of bromide in whole blood was approximately 12 per cent less than in serum. Conversion can be made by multiplying the results obtained from whole blood by 1.14 without introducing an error of much more than ±4 per cent. In the extreme instance as that calculated above from the assumed distribution between serum and corpuscles in marked anemia (hematocrit reading 20), the error would be increased to approximately 8 per cent.

CONCLUSIONS

1. The principles of determining bromides in biological materials are discussed critically.

2. A rapid and accurate method is described for determining both bromides and chlorides on a single sample of blood or tissue. Acid digestion is used and the silver halides are converted to soluble halides by treatment with thiosulfate.

3. A rapid and reliable clinical method is described by which bromide can be determined directly in 0.1 c.c. of blood drawn from a needle prick. The method is more accurate than any clinical method previously described and avoids the necessity for venepuncture.

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THE DETERMINATION OF DEMEROL IN URINE WITH PRELIMINARY OBSERVATIONS ON ITS EXCRETION IN MAN*

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CONSIDERABLE interest has arisen concerning the analgesic and antispasmodic properties of demerol which is the hydrochloride of 1-methyl-4-phenyl-piperidine-4-carboxylic acid ethyl ester. A number of reports have already appeared concerning its action on various physiologic functions, both in animals and in man.[1-8] To facilitate the study of the fate and manner of disposal of demerol in the animal body a method is suggested for determining demerol in urine, and some preliminary data are included on the urinary elimination of the drug in man.

The method depends upon the formation of a benzene-soluble compound between the free base of demerol and the dye bromthymol blue (dibromthymolsulfonphthalein) in an equimolar ratio. The free base is first extracted from the urine with benzene at pH 7.5. The resulting benzene solution of demerol is then shaken with an aqueous solution of the dye, also buffered at pH 7.5, whereupon an amount of dye approximately equivalent to the amount of demerol is carried into the benzene as a yellow compound.† This compound is readily decomposed so that the sodium salt of bromthymol blue may be extracted by aqueous alkali while the free base of demerol remains in the benzene and is discarded. The amount of dye extracted may be determined colorimetrically, and thus, indirectly, the original concentration of demerol.

PROCEDURE

The following solutions are required:

1. Concentrated buffer solution: 3.68 Gm. of $NaH_2PO_4 \cdot H_2O$ and 38.2 Gm. of $Na_2HPO_4 \cdot 12\,H_2O$ are dissolved in distilled water and made up to one liter.

2. Dilute buffer solution: 250 c.c. of solution 1 is diluted with an equal volume of distilled water.

3. Stock bromthymol blue solution: 0.400 Gm. of bromthymol blue (Eastman) is dissolved with warming in 30 c.c. of water and 6.4 c.c. of 0.100 N sodium hydroxide and made up to 100 c.c.

4. Dilute bromthymol blue solution: Solution 3 is diluted 1:5 with distilled water.

5. Buffered bromthymol blue solution: 40.0 c.c. of solution 4 are made up to 100 c.c. with solution 2.

The first operation is to buffer the urine to approximately pH 7.5. For this purpose a preliminary test is made. A drop of the stock bromthymol blue solu-

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tion is added to a mixture of 10 c.c. of concentrated buffer solution and 10 c.c. of the urine to be analyzed. The color of the resulting solution should be greenish blue to blue. If it is yellowish green to green, about 0.5 N sodium hydroxide solution is added drop-wise from a burette until the greenish-blue shade is produced. The indicated amount of sodium hydroxide is later added, in addition to the buffer, to fresh 10 c.c. aliquots of urine for analysis. If the urine contains a suspended precipitate, as is often the case, the test mixture should be centrifuged and the color of the supernatant liquid observed.

Three 250 c.c. separatory funnels are required for each determination. Ten cubic centimeters of concentrated buffer solution, 10 c.c. of the urine to be analyzed, and the proper amount of 0.5 N sodium hydroxide, as determined by the preliminary test, are placed in the first funnel, and enough water is added to bring the volume to 25 c.c. The demerol is extracted from the aqueous layer by shaking for thirty seconds with 60 c.c. of benzene. The aqueous layer is drained into the second funnel while the yellow benzene layer is decanted through the neck into the third funnel which must be dry.* The extraction is repeated twice with 50 c.c. portions of benzene, which are added to the first extract. The combined extracts are then shaken for two minutes with 25 c.c. of buffered bromthymol blue solution. The aqueous layer is drained into a flask and the benzene layer is decanted into a dry Erlenmeyer flask. The bromthymol blue solution is returned to the same funnel and is shaken gently with 35 c.c. of fresh benzene. Too vigorous shaking will result in an emulsion which separates only after long standing. The aqueous layer is now discarded, and the benzene extract is combined with the main portion which is allowed to stand until crystal clear. One to two hours is usually sufficient. The combined benzene extract is now decanted into a clean separatory funnel and extracted twice with 40 c.c. portions of 0.05 N sodium hydroxide solution, and the combined alkaline extracts are made up to 100 c.c. with 0.05 N sodium hydroxide. However, if the amount of demerol present in the original 10 c.c. of urine did not exceed 0.1 mg., it is preferable to extract the benzene solution with two 20 c.c. portions of 0.05 N sodium hydroxide and make the combined extracts up to 50 c.c. With practice, one can readily tell from the depth of yellow color in the benzene whether or not the amount of demerol in the original 10 c.c. of urine was greater than 0.1 mg.

The percentage transmission of the blue solutions is determined in a photoelectric colorimeter using a Corning filter combination of No. 350 (H.R. Traffic Yellow) and No. 398 (Dark Aklo).† The amount of demerol in the original 10 c.c. of urine is then determined by reference to a curve (Fig. 1) relating the log percentage transmission of the final blue solutions to the milligrams of demerol per 10 c.c. of urine. This curve was obtained by analyzing 10 c.c. aqueous samples containing appropriate amounts of demerol in place of urine in ex-

*In order to obtain benzene solutions practically free of the aqueous solution, each separation throughout the procedure is made as follows: the aqueous layer is drained through the stem of the funnel, and the funnel is rotated briskly to bring about a further separation of aqueous solution which, together with the major portion of any emulsion formed at the interface, is also drained through the stem. The benzene is then decanted through the neck of the funnel in such a way that traces of emulsion clinging to the sides of the funnel are retained on the walls.

†The absorption maximum of an alkaline bromthymol blue solution occurs at 6,170 angstrom units. This filter combination cuts off sharply at 5,400 angstrom units. Other combinations of filters were tried but none gave a steeper or more nearly linear curve of Beer's law.

actly the procedure described above. It will be seen from Fig. 1 that the curve follows the Lambert-Beer law in the range zero to 0.400 mg. of demerol, and deviates only slightly over the rest of the range.

It would appear that a curve based directly upon standard solutions of bromthymol blue might be used rather than the empirical curve (Fig. 1) for which each point must be obtained by the complete extraction process. This is not the case, however, because the theoretical amounts of bromthymol blue are obtained only approximately in the extraction process. The per cent of the actual amount of bromthymol blue obtained in the final extract to the theoretical amount expected was found to change progressively with the demerol content

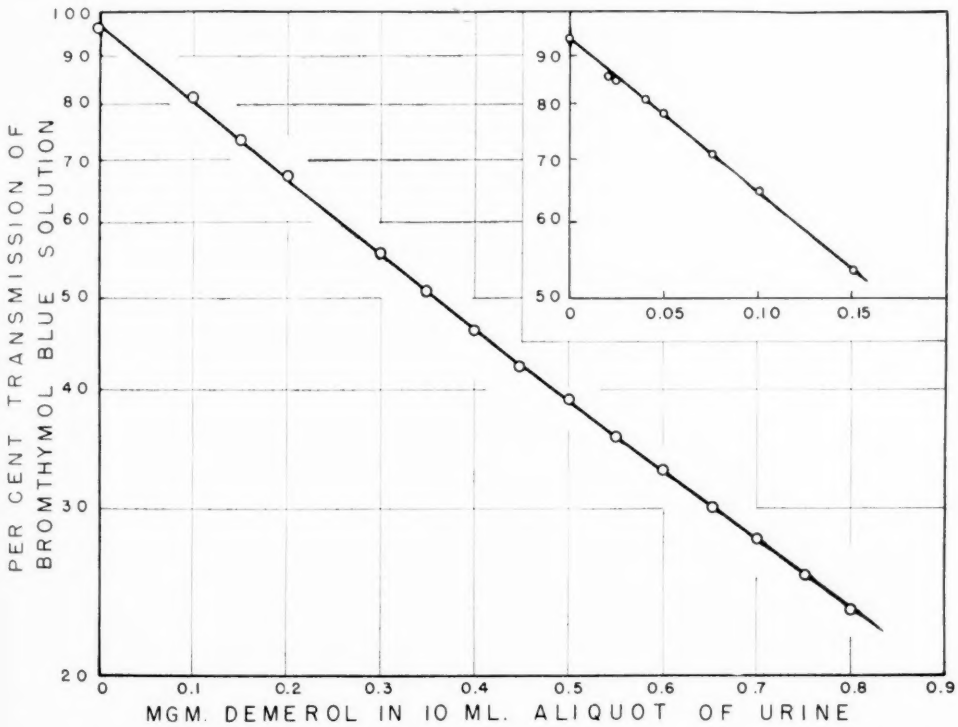

Fig. 1.—Relationship between the percentage transmission of bromthymol blue solution and the amount of demerol present in the original 10 c.c. sample of urine or aqueous solution. The inset shows the same relationship in the case where the final bromthymol blue solution has been diluted to 50 c.c. instead of 100 c.c.

of the original aqueous solution, as shown in Table I. The probable reasons for this deviation from theoretical are: (1) incomplete extraction of demerol base in the first step of the procedure; (2) loss of demerol into the aqueous layer in the second step, this being governed by the concentration of the excess dye, which, of course, is dependent upon the concentration of demerol; (3) extraction by the benzene layer of some uncombined dye present as a result of hydrolysis of sodium bromthymol blue in the buffered solution, the amount, which is small, being directly proportional to the amount of dye and indirectly proportional to the amount of demerol; and (4) the mechanical transfer of dye in the last step of the procedure. The curve does not pass through the origin, due to reasons (3) and (4).

TABLE I

VARIATION IN THE PER CENT OF A MOLAR EQUIVALENT OF BROMTHYMOL BLUE FOUND FOR DIFFERENT CONCENTRATIONS OF DEMEROL

CONCENTRATION OF DEMEROL TAKEN (MG./10 C.C.)	PER CENT OF A MOLAR EQUIVALENT OF BROMTHYMOL BLUE FOUND IN FINAL EXTRACT
0.05	120
0.10	100
0.15	97
0.20	93
0.25	92
0.30	92
0.40	89
0.50	88
0.60	86
0.70	87
0.80	86

TABLE II

RECOVERY OF DEMEROL FROM AQUEOUS SOLUTIONS

Analysis of 10 c.c. samples

DEMEROL TAKEN (MG.)	DEMEROL FOUND (MG.)	PER CENT ERROR
0.700	0.694	−0.9
0.700	0.699	−0.1
0.680	0.684	+0.6
0.650	0.651	+0.2
0.600	0.600	0
0.580	0.577	−0.5
0.550	0.550	0
0.500	0.503	+0.6
0.500	0.505	+1.0
0.450	0.451	+0.2
0.400	0.398	−0.5
0.350	0.351	+0.3
0.300	0.300	0
0.250	0.248	−0.8
0.200	0.201	+0.5
0.150	0.150	0
0.100	0.099	−1.0
0.075	0.072	−4.0
0.065	0.064	−1.5
0.060	0.057	−5.0
0.050	0.049	−2.0

RESULTS

Recoveries of demerol from aqueous solutions are shown in Table II. When the procedure was first applied to urine, it was noted that fresh alkaline urines (twenty-four-hour specimens) have a considerable blank, or apparent demerol concentration, while fresh acid urines have little or none. Old urines which have become alkaline on standing also show a blank. Since blanks occur in alkaline urines and not to any great extent in acid urines, it seems likely that the blanks are due to basic decomposition products. In order to study this factor a urine containing a known amount of demerol was aged under various conditions to show the effect of inhibiting decomposition with mercuric chloride, of allowing decomposition to occur normally, and of facilitating it by making the urine alkaline. Table III shows the results of this study. The demerol content of the portion of urine preserved with mercuric chloride did not change during the ten-day aging period, while the untreated portion had an apparent demerol

concentration of nearly twice the original value, and the portion made alkaline showed more than twice the original value.

Many organic bases react similarly to demerol, and the presence of such compounds in the urine will give rise to a blank or apparent demerol content. Quinine, codeine, aminopyrine, ephedrine, and nicotine have a demerol-like reaction, and it is likely that many other alkaloids do also. Morphine, however, in amounts likely to be present in the urine does not interfere seriously with the determination of demerol, since 10 mg. of morphine sulfate give an apparent demerol concentration of only about 0.1 mg.

TABLE III

EFFECT OF AGING UNDER VARIOUS CONDITIONS ON THE RECOVERY OF DEMEROL FROM URINE
Analysis of 10 c.c. samples containing 0.128 mg.

TREATMENT	DEMEROL FOUND (MG.)
Analyzed immediately	0.133 0.135
0.5 Gm. $HgCl_2$ added, analyzed after ten days	0.134 0.134
No treatment, analyzed after ten days	0.239 0.239
Made alkaline to litmus with solid $NaHCO_3$, and analyzed after ten days	0.283 0.292

TABLE IV

RECOVERIES OF DEMEROL ADDED TO URINES COLLECTED WITH MERCURIC CHLORIDE FROM SUBJECTS RECEIVING NO DRUGS

Analysis of 10 c.c. samples.

SUBJECT	DEMEROL TAKEN (MG.)	DEMEROL FOUND (MG.)	PER CENT ERROR
1	0.084	0.085	+1.2
	0.084	0.083	-1.2
2	0.113	0.114	+0.9
	0.113	0.114	+0.9
3	0.120	0.132	+10.0
	0.120	0.130	+8.3
4	0.400	0.400	0
	0.200	0.206	+3.0
5	0.400	0.401	+0.3
	0.200	0.194	-3.0
6	0.100	0.100	0
	0.325	0.325	0
7	0.093	0.096	+3.2
	0.093	0.094	+1.1
8	0.094	0.099	+5.3
	0.094	0.096	+2.1
9	0.300	0.324	+8.0
	0.300	0.327	+9.0
10	0.218	0.227	+4.1
	0.218	0.226	+3.7
11	0.134	0.133	-0.7
	0.134	0.137	+2.2
12	0.185	0.183	-1.1
	0.185	0.185	0
13	0.144	0.145	+0.7
	0.144	0.146	+1.4

Table IV shows recoveries of demerol added to normal twenty-four-hour urines which were collected in bottles containing 20 c.c. of saturated mercuric chloride solution from 13 subjects receiving no interfering drugs.

In order to determine the order of magnitude of the excretion of demerol, the drug in total doses of 100 to 200 mg. daily was administered to three subjects, and the twenty-four-hour urine specimens were analyzed by the method just described, using mercuric chloride as a preservative. The results are shown in Fig. 2. The data have been plotted in terms of per cent of the total administered demerol which had been recovered in the urine at any given time. It will

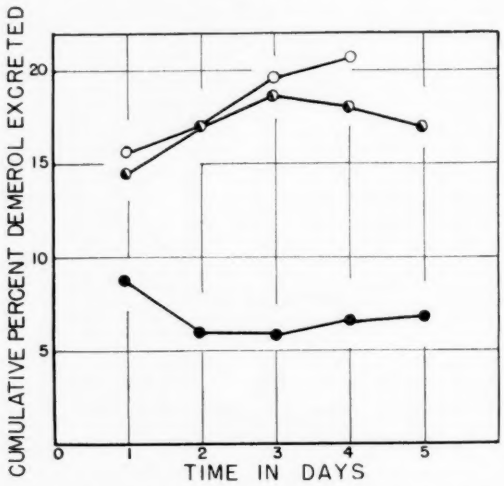

Fig. 2.—Excretion of demerol after oral administration of 100 to 200 mg. daily.

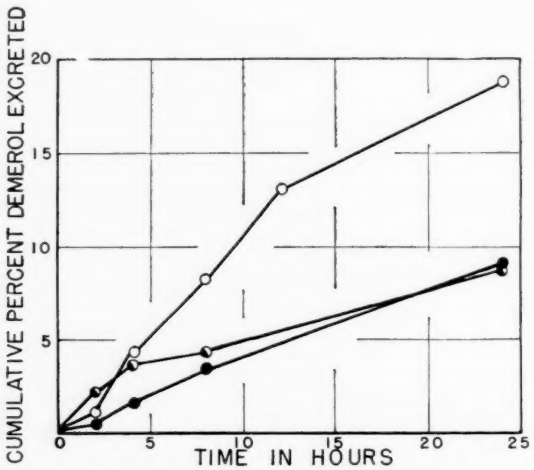

Fig. 3.—Excretion of demerol after subcutaneous injection of 100 mg.

be seen that, roughly, 5 to 10 per cent of the demerol is eliminated daily. To test the possibility that the demerol remaining unaccounted for may have escaped absorption in the intestinal tract, 100 mg. were administered subcutaneously to each of three subjects, and the urine was analyzed at intervals for the first twenty-four hours. The results of this study are shown in Fig. 3, and they agree in order of magnitude with those in Fig. 2. Thus, of 100 mg. injected, about 5 to 20 per cent has been excreted at the end of twenty-four hours. From

the agreement between the two routes of administration it seems probable that incomplete absorption is not accountable for the demerol which fails to appear in the urine.

SUMMARY

A method has been described by which demerol can be determined in amounts from 0.05 to 0.7 mg. per 10 c.c. of aqueous solution, with an average error of 0.9 per cent. The recoveries from urine indicate that amounts of demerol between 0.08 and 0.4 mg. per 10 c.c. (8 to 40 mg. per liter) can be determined, with an average error of 2.7 per cent. In no case did the error exceed 10 per cent.

Basic products resulting from the aging of urine cause a blank or apparent demerol concentration. Mercuric chloride prevents the formation of such products, and it is essential that it be used routinely in the collection of specimens.

The presence of various alkaloids in the urine interferes with the determination of demerol and care should be exercised accordingly in analyzing specimens when the subject has received other medication. Morphine does not interfere.

Preliminary excretion studies have been reported on six subjects who received therapeutic doses of demerol.

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BOOK NOTICES

Anoxia*

DR. VAN LIERE who has contributed a great deal to the subject of anoxia writes with justified authority. His book is primarily a discussion of the altered physiology of anoxia as it affects blood, tissues, cells, and the various body systems. There are relatively short chapters on mountain sickness and altitude sickness and acclimatization.

The volume will be of especial interest in aviation medicine, physiology, and biochemistry, and a helpful reference work for the clinician.

The Lymphatic System†

THE LYMPHATIC SYSTEM is a series of five lectures given at Stanford University in 1941. Dr. Drinker's first lecture discusses the evolution of the mammalian circulation. His second lecture is on the development and characteristics of the capillary circulation. The third lecture deals primarily with the lymphatic system. In the fourth, he discusses normal and abnormal physiology of the body fluids. The final lecture deals with practical problems in medicine and surgery.

Written by a world authority on the lymphatic system, this is essentially a practical summary which should be of value both to laboratory investigators and clinicians.

The Doctors Mayo‡

IT WAS to be anticipated that a biography of the Mayos, father and sons, would appear reasonably soon after the death of the last of this famous trio. These men represented the greatest achievement of private group practice that the world will probably ever see. While group practice will continue, it will undoubtedly be taken over increasingly by the state as a public service.

The contents of this book are sponsored by the University of Minnesota and are authentic. The volume is a little long for easy reading, but the most interesting part is the first half which describes the earlier formative years of the Mayo Clinic. The latter part of the book can be skimmed, and with considerable profit to the reader.

Manual of Clinical Chemistry§

REINER'S *Manual of Clinical Chemistry* is a small "pocket" book containing adequate directions for performing nearly all the clinical chemical examinations that are likely to be called for in hospital laboratories. Included are quantitative tests for the various hormones and vitamins; also toxicological tests, arsenic, lead, mercury, and barbiturates. There is one error of a decimal point on page 5 which, however, does not invalidate any of the procedures described in the book.

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*Anoxia. Its Effect on the Body. By Edward J. Van Liere, Ph.D., M.D. Cloth, 269 pages, $3.00. The University of Chicago Press, Chicago, Ill., 1942.

†Lane Medical Lectures: The Lymphatic System. Its Part in Regulating Composition and Volume of Tissue Fluid. By Cecil K. Drinker, Professor of Physiology and Dean of the School of Public Health, Harvard University. Stanford University Publications, University Series, Medical Sciences, Vol. IV, No. 2. Cloth, 101 pages, $2.25. Stanford University Press, Stanford University, California, 1942.

‡The Doctors Mayo. By Helen Clapesattle. Cloth, 822 pages, $3.75. The University of Minnesota Press, Minneapolis, 1941.

§Manual of Clinical Chemistry. By Miriam Reiner, M. Sc., Assistant Chemist to the Mt. Sinai Hospital, New York. Introduction by Harry Sobotka, Ph.D., Chemist to the Mt. Sinai Hospital, New York. Cloth, 296 pages, with 18 illustrations, $3.00. Interscience Publishers, Inc., New York, 1941.

Neuroanatomy*

DR. METTLER'S book is primarily a student text. The first portion deals with gross anatomy; the second, with microscopic anatomy. The book is so organized that when the medical student who has used it in his first year anatomy reaches his third and fourth year work in clinical neurology, he will find it a very handy reference volume. It is abundantly and well illustrated.

Pediatric Gynecology†

SIXTEEN years ago Dr. Schauffler, faced with a clinical problem of disease involving the genitals in a female child, found to his surprise that discussions of the general subject in the medical literature were few and rather noninformative. The present volume presents the results of his studies in the ensuing years. It is entirely a clinical book. It deals with methods of examination and treatment of disease of the sexual organs and other pelvic pathology in female children. The discussion carries on through abnormalities of adolescence. Diseases discussed include vaginitis, pelvic peritonitis, enuresis, pyelitis, rectal pathology, dysmenorrhea, and a large number of other conditions. There are sections on social service aspects of the treatment and supervision of these children; also on medico-legal aspects, in which is included a discussion of rape. The appendix includes lists of standard hormone preparations.

The work is practical and authoritative.

The Physiology of Domestic Animals‡

THE Physiology of Domestic Animals will be of greater use to veterinary physicians than to those dealing with human disease. However, it will be of distinct value to those who are interested in comparative physiology and to those in the field of experimental medicine who deal with animals in their work.

Methods of Treatment§

IT WOULD be rather difficult to furnish "an outline of all the methods of treatment in internal medicine" in a single volume of less than a thousand pages. Dr. Clendening has gone far toward accomplishing this. In succeeding editions the coverage has been increasingly complete. Naturally, the space devoted to any one subject is decidedly limited and the treatment is at times superficial. However, the volume serves very nicely as an index to treatment and as such makes a very adequate quick reference volume.

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*Neuroanatomy. By Fred A. Mettler, A.M., M.D., Ph.D., Professor of Anatomy, University of Georgia School of Medicine, Augusta, Ga. Cloth, 476 pages, with 337 illustrations, including 30 in color. The C. V. Mosby Company, St. Louis, 1942.

†Pediatric Gynecology. By Goodrich C. Schauffler, A.B., M.D., Assistant Clinical Professor of Obstetrics and Gynecology, University of Oregon Medical School; Visiting Gynecological Surgeon and Obstetrician, Multnomah Hospital, Portland, Ore. Cloth, 384 pages, $5.00. The Year Book Publishers, Inc., Chicago, Ill., 1942.

‡The Physiology of Domestic Animals. By H. H. Dukes, D.V.M., M.S., Professor of Veterinary Physiology, New York State Veterinary College, Cornell University. With a chapter on the Physico-Chemical Basis of Physiological Phenomena by E. A. Hewitt, D.V.M., Ph.D., Associate Professor of Veterinary Physiology, Division of Veterinary Medicine, Iowa State College; a Part on the Endocrine Organs and Reproduction, revised by S. A. Asdell, M.A., Ph.D., Professor of Animal Physiology, New York State College of Agriculture, Cornell University; and a Foreword by H. D. Bergman, D.V.M., Professor of Veterinary Physiology and Pharmacology, Division of Veterinary Medicine, Iowa State College. Cloth, ed. 5, revised, 721 pages, $6.00. Comstock Publishing Company, Inc., Ithaca and New York, 1942.

§Methods of Treatment. By Logan Clendening, M.D., Clinical Professor of Medicine, Medical Department of the University of Kansas; Attending Physician, University of Kansas Hospitals; and Edward H. Hashinger, A.B., M.D., Clinical Professor of Medicine, Medical Department of the University of Kansas; Attending Physician, University of Kansas Hospitals; Attending Physician, St. Luke's Hospital, Kansas City, Mo. With Chapters on Special Subjects by J. B. Cowherd, M.D.; Leland F. Glaser, M.D.; Thomas B. Hall, M.D.; John S. Knight, M.D.; H. P. Kuhn, M.D.; Paul H. Lorhan, M.D.; F. C. Neff, M.D.; Don Carlos Peete, M.D.; Carl O. Rickter, M.D.; E. H. Skinner, M.D.; O. R. Withers, M.D.; and Lawrence E. Wood, M.D. Cloth, ed. 7, 997 pages, The C. V. Mosby Company, St. Louis, 1941.

Disturbance of the Blood Coagulation in Infants*

THE well-known Swiss pediatrician, Fanconi, reports and discusses the carefully taken history of 47 infants suffering from various disturbances of the blood coagulation mechanism. Coagulation and bleeding time, prothrombin time, fibrinogen content, heparin content, calcium content, differential blood count, platelet count, etc., were determined by the most modern methods throughout the whole course of clinical observation. The cases are grouped under five main classifications: lack of fibrinogen, lack of calcium, lack of thrombokinase, hyperproduction of heparin, lack of vitamin K. The latter group is by far the largest. However, Fanconi emphasizes that most hemorrhagic diseases of the newborn are not caused by a single factor, but result from the interaction of various factors. Each time a new scientific discovery is made, its importance is overestimated. Thus, in consequence of the good results with vitamin K therapy, the K-avitaminose has been overrated as the sole cause of most hemorrhagic diseases in infants. An extensive bibliography increases the value of this small but very interesting book.

Ernst Fischer

Blood Disorders in Children†

WELL known, not only as a pediatrician, but also as an investigator, Dr. Kugelmass in this volume presents an authoritative and comprehensive discussion of a subject of importance, not only to the pediatrician, hematologist, and clinical pathologist, but also to the clinician at large.

The arrangement of the book follows the clinical rather than the hematologic viewpoint, and thus evaluates the blood picture in terms of the patient as a whole. A blood glossary and index of differential diagnosis based on both clinical picture and blood findings greatly enhance the usefulness of the book.

An outstanding contribution of great and general interest.

ITEM

The Laboratory of Mt. Sinai Hospital, Cleveland, Ohio, is making blood studies in suspected and known cases of trichinosis and shall appreciate it if readers of THE JOURNAL OF LABORATORY AND CLINICAL MEDICINE send whole clotted blood samples in such cases to Dr. B. S. Kline of that laboratory.

SIMPSON MEMORIAL INSTITUTE
ANN ARBOR, MICH.

*Disturbance of the Blood Coagulation in Infants With Special Reference to the Vitamin K and to the Pathology of the Newborn (Die Störungen der Blutgerinnung beim Kinde mit besonderer Berücksichtigung des K-Vitamins und der Neugeborenenpathologie.) By G. Fanconi. 160 pages, 21 figures. Georg Thieme, Leipzig, 1941.

†Blood Disorders in Children. By I. Newton Kugelmass, M.D., Ph.D., Sc.D., Spec. Attending Pediatrician, Downtown Hospital and Pan-American Clinics; Consultant Pediatrician, Heckscher Institute for Child Health, New York City Children's Hospital; Lynn Memorial Hospital, Monmouth Memorial Hospital, Muhlenberg Hospital; former Director of Pediatric Research, Fifth Avenue and Research Associate in Pediatrics, Yale University. Cloth, 897 pages, profusely illustrated, $10.00. Oxford University Press, New York.